Effects of Various Dietary Animal and Vegetable Proteins on Serum and Biliary Lipids and on Gallstone Formation in the Hamster[1]

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ABSTRACT

The objective of this study was to determine the effects of various dietary animal (casein, bovine albumin and egg albumin) and vegetable (soy, cottonseed and peanut) proteins on serum and biliary constituents and gallstone formation in the hamster. Eighty-four hamsters (60±5g) were assigned to either a control group (Purina rat chow) or to one of the 6 experimental groups. Experimental diets contained 20.0% protein. With the exception of hamsters fed egg albumin, gallstone incidence was greater among hamsters fed animal proteins. Hamsters fed egg albumin exhibited a lower concentration of total serum cholesterol and HDL-cholesterol than most of the other experimental groups. There were no significant differences between experimental groups for either HDL_3-cholesterol concentration or VLDL-LDL-cholesterol concentration. Bile acid concentrations within the vegetable protein-fed groups were significantly higher than within the animal protein-fed groups. Casein- and bovine albumin-fed hamsters showed a significantly higher percentage of biliary cholesterol in the bile fluid. As the percentage of biliary cholesterol increased, the percentage of bile acids was found to decrease. *Lipids* 20:1-6, 1985.

INTRODUCTION

Cholesterol gallstone disease occurs frequently in European and North American populations. Presently the etiology of this disease is poorly understood. However, it is known that the formation of gallstones involves supersaturation of bile with cholesterol. It is not known if this gallstone-inducing biliary cholesterol originates in the serum or if it is synthesized by the hepatic tissue.

Various dietary factors have been implicated in gallstone formation. Dietary cholesterol (1,2), fat (3,4) and carbohydrate (5,6) as well as excessive caloric intake (7) all have been reported to be involved in the development of gallstone disease. More recently, emphasis has been placed on the effects of dietary proteins on gallstone formation (8,9). In 1979 Kritchevsky and Klurfeld (8) demonstrated that when hamsters were fed the "Dam Diet" (a diet which contains no essential fatty acids, has sucrose as a carbohydrate source and uses casein as a protein source) as compared to a similar diet which contained soy protein, there was an increased incidence of cholesterol gallstones. Upon administration of a diet containing vegetable protein (soybean) to the cholelithic animals, gallstones were dissolved. More recently, Kritchevsky and Klurfeld (9) and Kritchevsky et al. (10) further documented the lithogenicity of casein as compared to soy protein.

The mechanism by which dietary proteins infuence cholesterol gallstone formation has not been determined. However, Kritchevsky (10) has shown that the lysine/arginine ratio of the diet can account for at least a portion of this diet-induced cholelithic effect. Earlier work by Liepa and Park (11) has shown that the lysine/arginine ratio of dietary protein has a significant impact upon serum cholesterol metabolism in rats. However, a relationship between serum and biliary cholesterol concentrations has not been studied extensively (12). Dietary proteins have been shown to alter serum concentrations of both total cholesterol (13,14) and various cholesterol lipoprotein fractions (15,16). Work done using both humans (13,14) and experimental animals (17,18) has indicated that animal proteins tend to be hypercholesterolemic, whereas vegetable proteins tend to be hypocholesterolemic. Although total serum cholesterol levels have not been associated with gallstone formation, some studies have shown correlations between various cholesterol lipoprotein fractions and cholelithiasis. Increased serum concentrations of very low density lipoprotein (VLDL) and low density cholesterol lipoprotein (LDL) have been associated with gallstone formation (19). Conversely, HDL-cholesterol concentration has been associated negatively with gallstone disease (20). Recent work by Portman et al. (21) showed a relationship between the concentration of the HDL_2

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subfraction of cholesterol and gallstone formation in squirrel monkeys.

This study was designed to investigate how various animal (casein, bovine albumin and egg albumin) and vegetable (soy, cottonseed and peanut) proteins, with a wide range of lysine/arginine ratios, affect concentrations of a variety of serum and biliary lipids and to determine if these lipid concentrations are correlated to gallstone incidence.

MATERIALS AND METHODS

Eighty-four golden Syrian hamsters *(Mesocricetus auratus;* SASCO, Inc., Omaha, Nebraska) were used in this study. Upon arrival, the hamsters were housed in metal cages in groups of 3 animals per cage. The animals were 4 to 6 weeks old and weighed 60 ± 5 grams. Food and water were provided ad libitum throughout the study. Animals were weighed weekly. The room was well ventilated and artificially illuminated on a 12-hr cycle.

Water bottles and cages were autoclaved weekly to prevent bacterial contamination. Animals were fed Purina Chow #5012 (Ralston Purina Co., St. Louis, Missouri) during a 14-day equilibration period and then randomly assigned to either an independent control group or one of 6 experimental groups (N = 12). The independent control group was maintained on the Purina Chow for the duration of the experiment. Animals given experimental diets were fed modifications of the Dam Diet (Table 1) which were mixed and pelleted by U.S. Biochemical Corporation (Cleveland, Ohio). The 6 experimental diets contained 20% protein by weight. Three different animal proteins (casein, bovine albumin and egg albumin) were used. All animal proteins were obtained from U.S. Biochemical Corporation, and contained 85.5%, 95.75% and 80.0% protein, respectively. Three different vegetable proteins (soy, cottonseed and peanut) also were used. The soy protein was supplied by U.S. Biochemical Corporation and contained approximately 90.0% protein. Cottonseed and peanut proteins were supplied by the Oilseed Protein Research Center at Texas A & M University, College Station, Texas, and contained 87.7% and 89.6% protein, respectively. The non-protein fraction of these experimental diets consisted of 74.3% sucrose and 5.7% vitamin/mineral supplements.

Hamsters were maintained on experimental diets for 63 days and then were fasted for 12 hr (receiving only water) and killed (9-11 a.m.). Hamsters were anesthetized with ether, abdominal cavities were opened and blood was aspirated via cardiac puncture. Blood samples were centrifuged for 20 min at 4,000 rpm at 4 C.

TABLE 1

Composition of Experimental Diets

Diet	Protein isolate source[3,4] (20% of diet)	Additional dietary components of all experimental diets
Experimental		
1	Casein[1(a)]	74.3% Sucrose
2	Bovine albumin[1(b)]	5.0% Mineral mix
3	Egg albumin[1(c)]	0.5% Vitamin mix
4	Soybean[1(d)]	0.2% Choline chloride
5	Cottonseed[2(a)]	
6	Peanut[2(b)]	
Control		
7	Purina Chow (5012)	

[1]Supplied by U.S. Biochemical Corporation, Cleveland, Ohio, and containing the following: (a) Casein–protein, 85.5%; moisture, 9.8%; ash, 1.8%; carbohydrate, 1.8%; fat, 1.0%; fiber, 0.1%; (b) Bovine albumin–protein, 95.75%; moisture, 2.4%; ash, 1.85%; (c) Egg albumin–protein, 80.0%; moisture, 8.0%; fat, 0.04%; carbohydrate, 0.1%; fiber-ash, 11.86%. Note: Biotin added at the rate of 2 mg/kg diet above biotin in vitamin mix; (d) Soy–protein, 86.0%; moisture, 5.5%; ash, 3.8%; carbohydrate, 3.8%; fat, 0.8%; fiber, 0.1%.

[2]Supplied by the Oilseed Protein Research Center, Texas A & M University, College Station, Texas, and containing the following: (a) Cottonseed–protein, 84.8%; ash, 6.5%; carbohydrate, 3.8%; moisture, 3.7%; fat, 0.6%; fiber, 0.6%; (b) Peanut–protein, 89.62%; moisture, 3.78%; carbohydrate, 3.31%; ash, 2.75%; fat, 0.40%; fiber, 0.14%.

[3]L/A ratios (casein, 2.51; bovine albumin, 1.22; egg albumin, 1.18; soy, 0.81; cottonseed, 0.30; peanut, 0.79; chow, 0.80).

[4]Glycine as per cent of total diet (casein, 0.30%; bovine albumin, 0.28%; egg albumin, 0.70%; soy, 0.84%; cottonseed, 0.91%; peanut, 0.94%; chow, 1.03%).

[5]Mineral and vitamin mixtures were identical to those used in Reference 12.

Serum was collected after centrifugation and was then refrigerated. Analysis of serum lipoprotein fractions was performed within 24 hr following collection, whereas analysis of total cholesterol concentration was performed within 72 hr. The gallbladder was examined visually for the presence of gallstones. Gallbladder bile was aspirated from the gallbladder, frozen and stored at -20 C for later chemical analysis. Total biliary bile acids, phospholipids and cholesterol were analyzed using the methods of Turley and Dietschy (22), Trudinger (23), and Reyes and Kern (24), respectively. Total serum cholesterol concentration was determined using the enzymatic technique described by Allain et al. (25). Serum lipoprotein cholesterol fractions were analyzed using the method of Warnick and Albers (26), whereas lipoprotein subfractions were determined using a modification of the method of Gidez et al. (27) which involved ultracentrifugation with a Beckman Airfuge (18 degree rotor).

Data were analyzed with a one-way analysis of variance for each variable examined. The error mean square was used as the basis for variability in subsequent tests. When a significant effect of dietary treatment was detected, the Scheffe procedure was used for selected contrasts between dietary groups, while the Newman-Keuls test was used for pairwise comparisons among groups (28).

RESULTS

Effects of Diet on Gallstone Incidence

In this study, gallstone incidence was not found to be strictly related to the source (animal vs vegetable) of the dietary protein. Among animal protein-fed groups, both bovine albumin- and casein-fed hamsters exhibited a significantly greater incidence (33% and 25%, respectively) of gallstones than any other groups of animals (Table 2). Hamsters fed either egg albumin, vegetable proteins or the control diet rarely exhibited gallstones (only one animal in all of these groups developed gallstones). There was no apparent relationship observed between weight gain and incidence of gallstone formation.

TABLE 2

Effect of Various Dietary Proteins on Animal Survival and Incidence of Gallstones

Group	Protein source	Survival	Gallstone incidence (%)
1	Casein	12/12	25.0
2	Bovine albumin	12/12	33.3
3	Egg albumin	12/12	0.0
4	Soy	12/12	0.0
5	Cottonseed	12/12	8.3
6	Peanut	12/12	0.0
7	Control	12/12	0.0

Effects of Diet on Serum Lipid Levels

When the effect of the various dietary proteins on total serum cholesterol and a number of cholesterol fractions and subfractions was analyzed, only hamsters fed egg albumin had a significantly lower ($P < 0.05$) concentration of total cholesterol (Table 3). When cholesterol

TABLE 3

Effect of Dietary Protein on the Concentration of Serum Cholesterol Constituents in Hamsters

Group	Protein source	Total serum cholesterol (mg/dl)	Total HDL cholesterol (mg/dl)	HDL_2 cholesterol (mg/dl)	HDL_3 cholesterol (mg/dl)	VLDL-LDL cholesterol (mg/dl)
1	Casein	137.4 ± 7.9^{a} (N = 12)	97.8 ± 5.5^{a} (N = 12)	63.9 ± 5.5^{a,b,c} (N = 12)	33.9 ± 4.9 (N = 12)	39.7 ± 6.9 (N = 12)
2	Bovine albumin	124.0 ± 8.1^{a} (N = 12)	103.5 ± 7.2^{a} (N = 11)	75.2 ± 7.4^{b,c} (N = 11)	29.1 ± 5.5 (N = 11)	22.4 ± 4.0 (N = 11)
3	Egg albumin	95.7 ± 4.5^{b} (N = 11)	74.6 ± 6.8^{b,c} (N = 9)	53.4 ± 4.7^{a,b,d} (N = 11)	24.4 ± 6.0 (N = 9)	20.2 ± 5.3 (N = 9)
4	Soy	124.0 ± 6.2^{a} (N = 10)	101.4 ± 5.6^{a} (N = 9)	69.7 ± 4.7^{b,c} (N = 9)	31.7 ± 3.4 (N = 8)	22.6 ± 5.7 (N = 8)
5	Cottonseed	137.6 ± 6.8^{a} (N = 10)	107.1 ± 5.3^{a} (N = 10)	73.3 ± 8.3^{b,c} (N = 10)	36.4 ± 5.9 (N = 10)	29.9 ± 6.4 (N = 10)
6	Peanut	127.0 ± 7.2^{a} (N = 11)	92.2 ± 5.5^{a,c} (N = 11)	46.5 ± 5.7^{a,d} (N = 10)	45.4 ± 5.9 (N = 10)	34.8 ± 5.0 (N = 11)
7	Control	96.3 ± 5.2^{b} (N = 12)	65.0 ± 6.2^{b} (N = 11)	36.6 ± 5.9^{d} (N = 10)	29.5 ± 5.1 (N = 10)	31.3 ± 7.6 (N = 11)

Values are means ± SE.

Means followed by different superscripts within a column are significantly different ($p < 0.05$).

TABLE 4

Effect of Dietary Protein on Biliary Composition of Hamsters

Group	Protein source	Absolute concentration (μmol/ml)			Relative concentration (molar %)		
		Bile acid[1]	Phospholipid[1]	Cholesterol[1]	Bile acid[1]	Phospholipid[1]	Cholesterol[1]
1	Casein	151.8 ± 9.7[a] (N = 12)	17.1 ± .2[a] (N = 12)	6.0 ± .9[a] (N = 10)	86.8 ± 0.4[a] (N = 10)	9.8 ± 0.4[a] (N = 10)	3.3 ± 0.3[a] (N = 10)
2	Bovine albumin	107.0 ± 7.5[b] (N = 11)	17.4 ± .6[a] (N = 11)	4.3 ± .6[a,b] (N = 10)	82.6 ± 0.6[b] (N = 10)	14.0 ± 0.7[b] (N = 10)	3.2 ± 0.3[a] (N = 10)
3	Egg albumin	155.3 ± 7.0[a] (N = 11)	12.3 ± .7[b] (N = 10)	2.3 ± .3[b] (N = 9)	91.3 ± 0.4[c] (N = 9)	7.3 ± 0.4[c] (N = 9)	1.3 ± 0.2[b,c] (N = 9)
4	Soy	232.6 ± 17.9[c] (N = 12)	16.6 ± .4[a] (N = 12)	4.3 ± .5[a,b] (N = 10)	91.8 ± 0.4[c,d] (N = 10)	6.5 ± 0.5[c,d] (N = 10)	1.5 ± 0.1[b,c] (N = 10)
5	Cotton-seed	241.1 ± 13.8[c] (N= 12)	14.4 ± .9[b] (N = 12)	4.7 ± .4[a,b] (N = 10)	92.5 ± 0.6[c,d] (N = 10)	5.4 ± 0.4[d,e] (N = 10)	1.8 ± 0.2[b] (N = 10)
6	Peanut	232.6 ± 14.5[c] (N = 11)	12.6 ± .7[b] (N = 11)	3.9 ± .6[a,b] (N = 11)	93.3 ± 0.4[d] (N = 11)	5.1 ± 0.3[d,e] (N = 11)	1.6 ± 0.2[b,c] (N = 11)
7	Control	279.3 ± 9.4[d] (N = 12)	12.5 ± .1[b] (N = 12)	2.8 ± .40[b] (N = 11)	94.7 ± 0.4[e] (N = 11)	4.3 ± 0.3[e] (N = 11)	0.9 ± 0.1[c] (N = 11)

Values are means ± SE.

Means followed by different superscripts within a column are significantly different ($p < 0.05$).

[1]Comparison between Groups 1-3 and Groups 4-6 is significant at $p < 0.01$ within a column.

fractions and subfractions were analyzed, both egg albumin- and peanut protein-fed hamsters had significantly lower ($P < 0.05$) total HDL-cholesterol and HDL_2-cholesterol concentrations than any of the other experimental groups. Although the casein- and chow-fed groups had decreased percentages of their total cholesterol in HDL, no relationship was apparent between per cent HDL (of total cholesterol) and gallstone incidence. There were no significant differences in VLDL-LDL or HDL_3 concentrations between experimental groups.

Effects of Diet on Biliary Lipid Levels

Bile fluid was analyzed quantitatively for the 3 primary lipid components, bile acids, phospholipids and cholesterol. Values were reported in absolute concentrations (μmol/ml) as well as in relative concentrations (molar %, Table 4). Bile acid concentrations within the vegetable protein-fed groups were significantly higher than within the animal protein-fed groups ($P < 0.05$). The bovine albumin-fed hamsters exhibited a significantly lower ($P < 0.05$) bile acid concentration than any of the other groups. The cottonseed protein-fed hamsters exhibited the highest concentration of bile acid of any of the experimental groups. The relative bile acid concentrations of bovine albumin- and casein-fed hamsters were significantly lower ($P < 0.05$) than the bile acid concentrations of any of the other groups.

The hamsters fed casein, bovine albumin and soy protein exhibited a significantly higher ($P < 0.05$) concentration of phospholipid than other experimental groups. The bovine albumin- and casein-fed hamsters exhibited the highest per cent phospholipid concentration ($P < 0.05$). The casein-fed hamsters exhibited the highest absolute and relative biliary cholesterol concentrations. Casein- and bovine albumin-fed hamsters showed the highest per cent biliary cholesterol concentration.

As the percentage of biliary cholesterol increased, the percentage of bile acids was found to decrease. A strong negative correlation ($r = -0.74$) existed between per cent biliary cholesterol and per cent bile acids. A stronger negative correlation ($r = -0.98$) existed between the molar % phospholipid concentration and the molar % concentration of bile acids.

DISCUSSION

Early work by Dam (29) indicated that hamsters fed dietary casein as part of the Dam Diet frequently developed gallstones. The present study did not completely support Dam's finding in that the incidence of gallstones in casein-fed animals was substantially lower than reported by Dam (28). The lower incidence of gallstones reported in the present study also has been observed by Kritchevsky et al. (9,10). The reasons for this are not absolutely clear. Optimum conditions for stone formation did exist (29). It is possible that provision of an infection-free environment through the research period altered stone formation since Dam had reported an increased incidence of intestinal disorders

and mortality in his study (29).

Effects of Dietary Proteins on Serum Cholesterol Profiles

A variety of studies (13,14,17,18) have shown that plant protein tends to have cholesterol-lowering properties in blood when compared to animal protein. In this study this was not found to be true. However, hamsters fed egg albumin had significantly lower serum cholesterol levels than those fed other dietary proteins. This egg albumin-induced hypocholesterolemia also has been shown to occur in rabbits fed egg white protein (30). In both animal (19) and human (20) studies, dietary animal protein (casein) also has been associated with increased concentrations of LDL- and VLDL-cholesterol, whereas diets containing vegetable (soy) protein have been associated with decreased concentrations of LDL-cholesterol and increased concentrations of HDL-cholesterol. Our study showed no differences in VLDL-LDL or HDL_3 concentrations between experimental groups. Although two dietary proteins (egg albumin and peanut) had significantly lower total HDL and HDL_2 cholesterol concentrations, no patterns were noted between lipoprotein differences and origin of dietary protein (animal vs vegetable). It is proposed that dietary protein-induced serum lipid changes observed in this study disagree with data presented in the literature possibly because of the uniqueness of the animal model (hamster) used. Data regarding effects of dietary proteins on serum lipid profiles of hamsters are not presently available for comparison.

Effects of Dietary Proteins on Biliary Lipids

According to Redinger and Small (31), gallstone formation is initiated when biliary cholesterol concentration is increased (above 5 molar %) in relation to the other biliary lipids. In this study the casein- and the bovine albumin-fed groups exhibited the highest relative concentrations of biliary cholesterol (3.3 molar % and 3.2 molar %, respectively) and also had the highest incidence of gallstones. Redinger and Small (31) further propose that should the concentration of biliary cholesterol cause the concentration of bile acids to fall below 80%, gallstone formation is initiated. The casein-fed hamsters as well as the bovine albumin-fed hamsters exhibited molar % concentrations of biliary bile acids (86.8 molar % and 82.7 molar %, respectively), which most closely approached the 80% limit proposed by Redinger and Small (31). Heaton et al. (32) proposed that gallstone formation is initiated by an elevation in the concentration of both biliary phospholipid and cholesterol. In the present study the casein- and bovine albumin-fed hamsters also exhibited the highest absolute and relative concentrations of biliary phospholipid.

Effects of Dietary Protein on Gallstone Formation

Recent work by Kritchevsky et al. (10) has shown that the lysine/arginine (L/A) ratio of experimental diets alters their lithogenicity. Casein was shown to be lithogenic in their study, but when arginine was added to the diet there was a significant decrease in gallstone incidence. Dietary soy protein, which was associated with a much lower incidence of gallstones, became more lithogenic when lysine was added to the diet. Although all of the animal protein diets used in our study had L/A ratios above 1.0 and vegetable protein diets had ratios below 1.0, egg albumin had a L/A ratio (1.18) which most closely approached vegetable protein ratios. Gallstones were found most frequently in hamsters fed diets containing either casein or bovine albumin. This pattern of stone incidence might be expected if the L/A ratio hypotheses proposed by Kritchevsky et al. (10) were true.

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Phospholipid Composition of Guinea Pig Lung Lavage

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ABSTRACT

Phospholipids from guinea pig lung lavage were analyzed. The total lavage phospholipid content was 2.65 + 0.67 mg per gram of lung, which accounted for 85% of the total lipids in lung wash. Phosphatidylcholine (PC) accounted for over 60% of the total phospholipids. The other phospholipid fractions, in order of predominance, were phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SPH), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and lysophosphatidylcholine (LPC). Disaturated phosphatidylcholine (DSPC) comprised 80% of the total PC, and it contained mostly palmitic acid. The DSPC content of the lung lavage fluid per square meter of alveolar surface area was 5.76 ± 0.42 mg.
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INTRODUCTION

The lung becomes functional at birth, when it undergoes a transition from a liquid to a gas filled environment. The transition cannot occur successfully in the absence of a surface-active material, commonly known as pulmonary surfactant, which promotes alveolar stability. Pulmonary surfactant is a complex, water-insoluble material consisting principally of lipids (85%), proteins (13%) and carbohydrates (3%) (1). Surfactant from several species studied (2-6) contains a large quantity of saturated PC (41-58% by weight of the total lipid), particularly dipalmitoylphosphatidylcholine (DPPC) (1). PG is also an essential component of pulmonary surfactant (7,8). Smaller amounts of other lipids, such as acidic phospholipids, PE and cholesterol are also present in the surfactant system. The surfactant lipids are believed to be synthesized in rough endoplasmic reticulum, stored in the lamellar bodies of the Type II alveolar epithelial cells and secreted into the alveoli as part of a complex mixture of lipids and proteins (5,9-11). Surfactant lipids from the alveoli and from cells can be isolated separately, the former by lavaging alveoli and the latter by analyzing lung tissue post-lavage (12) or by studying purified type II cells (13). Since the phospholipid composition of lung wash resembles that of lamellar bodies and surface-active material (3,14-18), the efficiency of the pulmonary surfactant system can be evaluated by the study of the lavage material.

In view of the difficulties in conducting such studies in man, a suitable animal model for surfactant secretion is essential. We have developed an animal model using the guinea pig in which to study the lung surfactant biosynthesis (19-22). It has been established that guinea pig lung is more like human lung tissue than other frequently-used animal lung tissues in the regulation of surfactant phospholipid synthesis and in fetal lung maturity (22). Unfortunately, no information is available on the composition of surfactant lipids in this species. This study presents a quantitative analysis of phospholipids from alveolar washings in the guinea pig.

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EXPERIMENTAL PROCEDURES

Preparation of Lavage

Three-week-old male Hartley guinea pigs weighing 250-275 g each were obtained from Camm Research Laboratories, New Jersey. Each animal was injected intraperitoneally with 400 U heparin and 10 mg pentobarbital/100 g b. wt. After the animals were anesthetized, the chest cavity was opened and the lungs perfused by gravity with a solution composed of 136 mM NaCl, 5.3 mM KCl, 5.6 mM glucose, 2.6 mM sodium phosphate buffer, 10 mM Hepes buffer, pH 7.4 (23). A cannula was inserted into the pulmonary artery via the right ventricle and the left auricle was cut to allow the blood to wash out of the lungs. During the perfusion, the lungs were ventilated manually with air through a cannula inserted into the trachea. Following thorough perfusion the lungs were removed intact from the thorax and lavaged 6 times via the cannulated bronchus, each time with 10 ml of cold perfusion solution. Preliminary experiments showed that approximately 99% of the saturated PC recovered after 6 separate lavages was found in the first 4 lavage returns. If blood was detected by gross examination in lavage fluid, the sample was rejected. Cellular components, chiefly alveolar macrophages, were removed by centrifugation at 800 × g for 10 min, and the super-

natant was stored in a freezer at −20 C until analysis. Lavaged lung was weighed after all extrapleural vessels and bronchi were carefully trimmed away. Protein content of lavage fluid was measured by the procedure of Lowry et al. (24).

Lipid Extraction, Fractionation and Analysis

Lipid was extracted from lung lavage fluid by the procedure of Bligh and Dyer (25). Crude lipid extracts were washed with potassium chloride (26) and freed from non-lipid contaminants by Sephadex column chromatography (27). A known aliquot of eluate from the Sephadex column was evaporated to dryness in a tared flask, and the weight of the lipid residue was determined.

Lipids were separated into neutral lipid, glycolipid and phospholipid fractions on a silicic acid column by the successive use of chloroform, acetone and methanol as the eluting solvents (28). To isolate DSPC, the phospholipids were reacted with osmium tetroxide dissolved in carbon tetrachloride and fractionated on a column of neutral alumina by the procedure of Mason et al. (29). Phospholipids were separated into individual components by thin-layer chromatography (TLC) (30). Phosphomolybdate sprays (31) were used as the visualizing agents to detect separated phospholipids. The quantity of total phospholipids in the methanol eluate and individual phospholipids isolated from the TLC plates was determined by measuring the amount of phosphorus according to the procedure of Bartlett (32). To determine the fatty acid composition of PC and DSPC, the lipids were saponified and the isolated fatty acids converted to methyl esters by heating for 5 min at 75 C with 14% boron trifluoride in methanol, according to the method of Metcalf and Schmitz (33). The methyl esters of fatty acids were separated on a 6 ft × ¼ in. column of 10% diethylene glycol succinate on Chromosorb W AW (80-100 mesh) in a Packard Model 7400 gas chromatograph equipped with flame ionization detector (34).

RESULTS

The mean weight of the animals was 265.2 ± 3.3 g. The mean wt of lung was 2.67 ± 0.32 g. Table 1 presents quantitative data for the total lipids, total and individual phospholipids and total protein in lung lavage fluid. The total lipid and protein content was 3.31 ± 0.53 mg and 0.36 ± 0.09 mg respectively, expressed as per gram of lung. Phospholipid, the major component of the total lipids, constituted 58% of the total lipids. PC constituted the largest fraction of lipids, comprising 64.5% of the total phospholipids. In order of predominance, PI, PS, SPH, PE, PG and LPC were the other major lipids, and comprised 6.4%, 6.1%, 6.1%, 5.2%, 4.0% and 4.0% of the total phospholipids, respectively. DSPC made up 79.9% of the total PC in lavage fluid.

Table 2 presents the composition of fatty acids of PC and DSPC from lavage fluid. Palmitic acid accounted for 68.0% and 88.1% of the total PC and DSPC, respectively, and there were few fatty acids with more than 18 carbon atoms. They contained relatively little stearic acid (5.1% for PC and 3.8% for DSPC). Saturated fatty acids accounted for 79.5% of the total in PC.

DISCUSSION

The composition of the phospholipids in

TABLE 1

Composition of Phospholipids and Proteins of Lung Lavage Fluid

	mg per g of wet lavaged lung (Mean ± SD, N = 8)	mg per sq meter of alveolar surface area (Mean + SD, N = 8)[a]
Total lipids	3.31 ± 0.53	14.02 ± 2.2
Total phospholipids	2.65 ± 0.67	11.23 ± 2.63
Phosphatidylcholine	1.71 ± 0.23	7.24 ± 0.97
Disaturated phosphatidylcholine	1.36 ± 0.19	5.76 ± 0.80
Phosphatidylinositol	0.17 ± 0.04	0.72 ± 0.16
Phosphatidylserine	0.16 ± 0.04	0.67 ± 0.15
Sphingomyelin	0.16 ± 0.05	0.67 ± 0.16
Phosphatidylethanolamine	0.14 ± 0.03	0.59 ± 0.09
Phosphatidylglycerol	0.11 ± 0.01	0.46 ± 0.09
Lysophosphatidylcholine	0.10 ± 0.01	0.42 ± 0.04
Total protein	0.36 ± 0.09	1.52 ± 0.38

[a]The alveolar surface area value is obtained from the literature (Tenney and Remmers, Nature, 197, 54-56).

TABLE 2

Fatty Acid Composition of Phosphatidylcholine and Disaturated Phosphatidylcholine of Lung Lavage Fluid

% of Total Fatty Acids (means ± S.D., N = 8)

Fatty acid	Phosphatidylcholine	Disaturated phosphatidylcholine
14:0	5.4 ± 0.4	6.8 ± 0.7
15:0	1.0 ± 0.1	1.3 ± 0.1
16:0	68.0 ± 3.9	88.1 ± 4.8
16:1	4.2 ± 0.5	
18:0	5.1 ± 0.7	3.8 ± 0.6
18:1	9.2 ± 1.2	
18:2	6.1 ± 0.9	
18:3	0.4 ± 0.0	
20:4	0.6 ± 0.1	

guinea pig lung washings are similar to those reported in other species, such as bovine (6), rabbit (7,35), dog (23,24,36), human (37), and mouse and rat (38,39). However, there is a significant quantitative difference in the phospholipid composition between guinea pig and other species, particularly bovine (6) and rabbit (35). Rooney, Page-Roberts and Motoyama (35) reported that PC and PG account for 86.2% and 6.2% of the total phospholipids, respectively, in adult rabbit lung wash; PE, PI, PS and SPH account for a total of only 6%. Similar results were obtained for bovine (6). However, in guinea pig lavage, PC and PG account for only 64.5% and 4.0% of the total phospholipids, respectively. PE, PI, PS and SPH account for more than 23% of the total phospholipids. The striking difference is that PG is the second major phospholipid in rabbit and bovine, whereas in guinea pig PI is the second major phospholipid.

The significance of this difference is not clear at this time. However, it may be noted that CDP-diglyceride serves as a common precursor for both PI and PG, and the latter compound seems to be an end product rather than a precursor (40). In the fetal rabbit, PI is the most abundant acidic surfactant phospholipid, but its level decreases after birth. On the other hand, PG is absent in surfactant from the fetal rabbit, appears at term and increases after birth (41). We do not know at this time what kind of developmental changes occur in the amount of these two acidic phospholipids in guinea pig lung. However, it should be noted that recently Beppu et al. (42) have reported that the presence of PG is not essential in the adult rabbit for normal in vitro surfactant properties. Their results suggest that PI may substitute for PG for maintaining normal surfactant structure.

Gail, Steinkamp and Massaro (39) reported that the amount of saturated PC in lung lavage shows a direct log linear correlation with species alveolar surface area at functional residual capacity. The saturated PC content of lung lavage fluid per square meter of alveolar surface area varies in the sequence: mouse (12.6 mg) > rat (6.2 mg) > rabbit (5.4 mg) ≥ dog (4.6 mg) > cat (3.1 mg). Their data indicate that the amount of lavage saturated PC per square meter of alveolar surface is larger in small animals with small alveoli than in larger animals with larger alveoli. Tenney and Remmers (43) have measured the alveolar surface area of various species and, according to their data, the alveolar surface area varies in the squence: dog (46.41 m^2) > cat and rabbit (7.29 m^2) > guinea pig (0.63 m^2) > rat (0.46 m^2) > mouse (0.06 m^2). If the DSPC content of guinea pig lavage is calculated on the basis of alveolar surface area data of Tenney and Remmers (43), then the guinea pig lavage contains a total of 5.76 mg DSPC per square meter of alveolar surface area. It is not surprising that DSPC in the lavage returns of guinea pig is closer to that of rabbits and rats as compared to larger animals (39). It is suggested that the curve established by Gail et al. (39) in relating lavage saturated PC and alveolar surface area can be applied successfully in computing the saturated PC content of lavage of any species of known alveolar surface area.

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Lipids of Dermatophytes II. Effect of Growth Condition on the Lipid Composition and Membrane Transport of *Microsporum gypseum*

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ABSTRACT

Supplementation of glucose-containing medium with ethanol and replacement of glucose by glycerol in the Sabouraud's growth medium of *Microsporum gypseum* altered the levels of total phospholipids as well as their apolar and polar head groups. The levels of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) increased under these growth conditions; also, the ratio of unsaturated/saturated phospholipid fatty acids decreased on ethanol supplementation but increased in the presence of glycerol. Steady state accumulation of labelled amino acids (glycine, lysine and aspartic acid) was affected under these conditions.

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INTRODUCTION

Dermatophytes are a group of pathogenic fungi infecting keratinized surfaces of animals including man. Since recognition that dermatophyte lipids are allergens to the host (1), work has been initiated to examine dermatophyte composition (2-5) and lipid metabolic pathways (6,7). Earlier studies from our laboratory have reported the lipid composition of these fungi (8) as well as their characterization and purification of key phospholipid metabolizing enzymes (9-12). In continuation of our studies on the lipid biochemistry of dermatophytes, we now have focussed our attention on the role of lipids in membranes. Investigations have not been undertaken to explore the effects of different growth conditions on dermatophyte lipids and the subsequent effects on membrane properties. In this study, *Microsporum gypseum*, a dermatophyte, was exposed first to Sabouraud's medium, supplemented with one per cent ethanol, a disinfectant-cum-membrane perturbant (13). Second, glycerol was substituted for glucose, the main carbon source in the growth medium. Glycerol was chosen because it is a natural carbon source available to the fungus during infection (14). The alterations produced in lipid species under these conditions have been shown to influence membrane transport functions of *M. gypseum*.

MATERIALS AND METHODS

Materials

Labelled amino acids (1-^{14}C-glycine, sp. activity 144 mCi/mmol; U-^{14}C-lysine of sp. activity 230 mCi/mmol and U-^{14}C-aspartic acid of sp. activity 152 mCi/mmol) were procured from BARC, Bombay, India. Malonyl-(2-^{14}C) coenzyme A (sp. activity 50.7 mCi/mmol) and Oleoyl-(1-^{14}C)-coenzyme A (sp. activity 60 mCi/mmol) were obtained from NEN, Massachusetts, USA. NADPH, ATP, oleoyl CoA, malonyl CoA, cycloheximide, glycine, aspartic acid, lysine were the products of Sigma Chemical Co., St. Louis, Missouri, USA. Ficoll paque was purchased from Pharmacia Fine Chemical Co., Sweden. Novozym and Cellulase 'CP' were gifts sent by M/s Novo Industrie, Bagswaerd, Denmark, and John & E. Sturge Ltd., Selby, North Yorkshire, England, respectively.

Growth of Culture

Microsporum gypseum, obtained from the Mycological Reference Laboratory, School of Hygiene and Tropical Medicine, London, England, was grown in Sabouraud's broth (4% glucose, 1% peptone, pH 5.4-5.6). Where indicated, one per cent ethanol was supplemented into the autoclaved medium under sterile conditions. However, for other experimental cultures glucose was replaced equivalently by glycerol (4%, w/v) in the Sabouraud's medium. The fungus was grown as surface cultures at 27 C until the logarithmic phase of growth was complete. The cells were harvested after 18 days of cultivation and were then processed for lipid extraction and enzyme assays, as per the following methods.

Quantitation of Lipids

Total lipids of the cells were extracted and purified by the method of Folch et al. (15). Total phospholipids were quantified by the

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standard method of Bartlett as modified by Marinetti (16). Two dimensional thin layer chromatography was used to separate total phospholipids into individual species by using the solvent systems: chloroform-methanol-water, 65:25:4 (v/v/v for the first dimension) and chloroform-methanol-water-acetic acid, 50:35:1:8 (v/v/v/v for the second dimension). Total phospholipids, separated from the neutral lipids, were analyzed for their fatty acyl composition by gas liquid chromatography (Pye Unicam Model 104) following their conversion into methyl esters as described by Khuller et al. (17).

Enzymes of Fatty Acid Biosynthesis (Fatty Acid Synthetase and Desaturase)

A 15 per cent homogenate from various cultures was prepared by crushing, homogenizing and sonicating the cells (at 4 C) suspended in 10 mM Tris-HCl buffer (pH 7.0). The homogenate was spun at 1000 x g for 15 min and the pellet, containing mainly cell debris, was discarded. The supernatant was centrifuged further for removal of mitochondria. The resulting supernatant was subjected to ultracentrifugation at 100,000 x g for 90 min. The supernatant was the source of fatty acid synthetase, whereas the pellet was the source of desaturase. The pellet was resuspended in 10 mM phosphate buffer (pH 7.1) prior to use.

Fatty Acid Synthetase

The method of Klein (18) was followed for assay of fatty acid synthetase. Following incubation at 30 C for 20 min, the reaction was terminated with 1 ml of 10% methanolic KOH. The mixture was saponified for one hr at 70 C and subsequently supplemented with 15 μg of middle and long chain fatty acids. Both cold and labelled fatty acids were extracted with pentane. The pentane extract was dried and the residue dissolved in a minimal volume of chloroform. Samples were counted in a Packard Scintillation Counter using toluene based scintillation fluid. Protein was determined by the method of Lowry et al. (19).

Desaturase Assay

The assay mixture was the same as described by Baker and Lynen (20). Incubation and termination of the reaction was the same as for the fatty acid synthetase assay. Fatty acids were extracted with pentane; the product and the substrate were separated by argentation chromatography (21). The spots, identified by spraying with Rhodamine 6G and visualized under an ultraviolet lamp, were scraped directly into counting vials and toluene scintillation fluid was added (10 ml/vial). Counts incorporated were measured in a liquid scintillation counter.

Preparation of Spheroplasts

After harvesting various types of cells under sterile conditions, a known portion (1-2 g) of the mycelium was added to a flask containing autoclaved solution A (consisting of 3 mM β-mercaptoethanol in 20 mM citrate phosphate buffer, pH 6.5). After incubation at 30 C for 60 min, solution A was decanted and 10 ml sterile solution B (3 mM dithiothreitol, 0.7 M NaCl and 10 mM citrate phosphate buffer, pH 6.5) and lytic enzymes (Novozym and cellulase 'CP,' 30 mg each) added. The incubation was carried out for 20 hr at 30 C, and the formation of spheroplasts was checked microscopically. The incubation mixture containing solution B and the enzymes was discarded. Solution B (20 ml) was added and the mixture was centrifuged at 1000 x g for 10 min. The spheroplast pellet was washed twice similarly with solution B for complete removal of the enzymes. The final suspension of spheroplasts was put on Ficoll paque gradient and centrifuged at 400 x g for 30-40 min at 18 C. The purified spheroplasts obtained were used for transport studies.

Transport Studies

Spheroplasts containing 100-150 μg protein were preincubated with 100 μg cycloheximide at 28 C for 10 min in solution B lacking dithiothreitol. 0.8 mM labelled amino acid (1-^{14}C-glycine/U-^{14}C-aspartic acid/U-^{14}C lysine) was added and the specific activity adjusted to 0.2 μCi/mmole in a total volume of 0.2 ml. The reaction was stopped after 5 min of incubation at 28 C by diluting it with chilled normal saline followed by filtration through 0.22 μm Millipore membrane filters. After 2-3 washes with chilled normal saline, the filter was dried completely and counted in toluene based scintillation fluid.

Statistical Analysis

Differences between various types of cells were calculated by Student's t-test.

RESULTS AND DISCUSSION

Phospholipids are constituents of biomembranes that affect diverse vital phenomena including membrane polarity, membrane fluidity and metabolite transport (22,23). Alterations in membrane properties following changes

in phospholipid composition, produced either by genetic manipulations or by changes in growth conditions, indicate a relation between structure and function of biomembranes.

As is evident from Table 1, content of total phospholipids (TPLs) increased appreciably both in ethanol supplemented and in glycerol grown cells. The enhancement was nearly two-fold in the latter type of cells compared to the control cells, which were grown in normal Sabouraud's medium devoid of any supplement. The changes observed in the total phospholipid levels also were reflected in their individual components (Table 1). The levels of both PC and PE were raised under both of the test culture conditions. The augmentation was 1.4- and 1.6-fold in PC and PE levels respectively, in the ethanol supplemented cells. However, the increase was more pronounced in the glycerol grown cells, where PC and PE levels were raised by 2.2- and 2.8-fold, respectively. Phosphatidylserine (PS) plus phosphatidylinositol (PI) levels were increased significantly in the glycerol grown cells (Table 1). Since a major proportion of the total cellular content of phospholipids generally is present in the membranes (24), the enhancement observed in the level of total phospholipids and the major phospholipid components (PC and PE) suggests adaptation of the dermatophyte membranes to their environment. Further, the ratio between zwitterionic (LPC + PC + PE) and anionic (PS + PI) species of lipids also was altered (Table 1) in both ethanol supplemented and glycerol grown cells of *M. gypseum*. Since charge on polar head groups of phospholipids is crucial for binding or neutralization of other polar membrane components (25), alterations in membrane polarity implicate changed membrane properties of *M. gypseum*.

Fatty acyl chain composition of phospholipids was altered markedly on ethanol supplementation (Table 2). Ethanol addition caused a 4-fold decrease in the content of linoleate ($C_{18:2}$) and an increase of 2- and 4-fold in the levels of palmitate ($C_{16:0}$) and stearate ($C_{18:0}$), respectively, resulting in a sharp decline in the ratio of unsaturated/saturated phospholipid fatty acids in ethanol exposed cells compared to the control. However, on substitution of glucose by glycerol, a significant increase was observed in the unsaturated/saturated fatty acid ratio (Table 2). This increase was accomplished by an increase in the levels of linoleate with a simultaneous decrease in palmitate in these cells, compared to the control. Since fatty acyl chains of phospholipids are known to regulate membrane fluidity and membrane functions (22), the alterations manifested following culti-

TABLE 1

Effect of Stress Conditions on Phospholipid Composition of *M. gypseum*

Cell type	Total phospholipids (mg PLs/g dry wt.)	Individual components (mg P.L./g dry weight)				Ratio of zwitterionic/ anionic phospholipids
		LPC	PC	PS + PI	PE	
Glucose (control)	10.75 ± 0.82	0.83 ± 0.20	4.05 ± 0.85	3.25 ± 0.25	2.61 ± 0.18	2.30
1% Ethanol	14.12 ± 0.52***	0.69 ± 0.21NS	5.74 ± 0.45**	3.57 ± 0.72NS	4.12 ± 0.49***	2.95
Glycerol	22.31 ± 0.64***	1.41 ± 0.33**	11.53 ± 1.21***	3.68 ± 0.36*	5.69 ± 0.62***	4.78

The values are Mean ± S.D. of 3 batches analyzed in duplicate.
NS = Non-significant; *$P < .05$; **$P < .01$; ***$P < .001$, compared to the control cells.

TABLE 2

Relative Proportion of Phospholipid Fatty Acids in Variously Grown *M. gypseum* Cells

Cell type	Relative percentage of fatty acids					u/s fatty acids ratio
	$C_{10:0} + C_{12:0} + C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	
Glucose (control)	1.21 ± 0.43	40.55 ± 1.65	t	3.14 ± 1.72	13.47 ± 0.83	40.94 ± 1.46	1.22 ± 0.08
1% Ethanol	0.73 ± 0.20*	61.10 ± 1.55***	t	11.75 ± 0.78***	16.64 ± 1.10***	9.78 ± 1.02***	0.36 ± 0.10***
Glycerol	1.33 ± 0.51NS	34.20 ± 0.72***	t	5.68 ± 0.79**	14.25 ± 0.78NS	44.52 ± 0.83***	1.43 ± 0.06***

Values are Mean ± S.D. of 6 batches analyzed in duplicate.
t = trace amounts; NS = Non-significant; *P < .05; **P < .01; ***P < .001, compared to the control cells.

vation under different growth conditions appear to be obligatory for survival of the fungus under these conditions.

To examine the effect of different growth conditions on the enzymes of fatty acid biosynthesis, activities of fatty acid synthetase and desaturase were determined in ethanol supplemented and glycerol grown cells. The results are presented in Table 3.

Because fungi possess fatty acid synthetase type I complex (27), responsible for synthesis of only saturated fatty acids, the stimulation observed in the specific activity of fatty acid synthetase from ethanol supplemented *M. gypseum* (Table 3) suggests it was responsible for decreasing the unsaturated/saturated fatty acids ratio in these cells. In spite of the enhancement of unsaturated phospholipids noted in glycerol grown cells, the unaffected activities of fatty acid synthetase and desaturase in such cells indicate probable participation of either acyltransferase (28,29) or direct phospholipid desaturase (30) in controlling the fatty acyl composition, as has been reported for other microorganisms.

Because alterations in lipid composition often have been documented to regulate membrane transport functions (31,32), membrane properties of various *M. gypseum* cultures were examined by studying the uptake of amino acids. It is pertinent to mention that the non-specific barrier, i.e. cell wall, of densely interwoven dermatophyte mycelia was removed enzymatically and that the resulting round, protoplasmic spheroplasts were used for the transport function studies. Three amino acids, glycine, lysine and aspartic acid, representing the non-polar, basic and acidic category of amino acids respectively, were selected for the transport studies.

As is evident from Table 4, spheroplasts

TABLE 3

Effect of Cultural Conditions on Activities of Fatty Acid Synthetase and Desaturase

Cell type	Fatty acid synthetase† (n moles/mg protein/20 min)	Desaturase
Glucose (control)	9.60 ± 2.2	4.14 ± 0.21
1% Ethanol	23.06 ± 2.7***	3.78 ± 0.35NS
Glycerol	9.68 ± 2.1NS	4.52 ± 0.17NS

Values are Mean ± S.D. of the 3 independent determinations.

†The reaction products were devoid of any unsaturated fatty acid.

NS = Non-significant; ***P < .001, compared to the control.

TABLE 4

Uptake† of Amino Acids by Spheroplasts Prepared from Variously Cultured *M. gypseum* Cells

Cell type	Glycine	Aspartic acid	Lysine
Control	54.19 ± 3.58	33.22 ± 2.14	33.04 ± 1.04
1% Ethanol	31.69 ± 1.54***	24.15 ± 1.21**	30.30 ± 0.34*
Glycerol	32.56 ± 1.39***	23.18 ± 1.19**	26.76 ± 0.97**

†Uptake = n moles a.a/mg protein/5 min. The values are Mean ± S.D. of 3 independent determinations.

*$P < 0.05$; **$P < .01$; ***$P < .001$.

NS = Non-significant compared to the control.

from both ethanol supplemented and glycerol grown cells showed significant decrease in steady state accumulation of all three amino acids compared to the control spheroplasts. The changes may be attributed to alterations observed in phospholipid composition, including those in the ratio between zwitterionic and anionic lipid species in ethanol supplemented and glycerol grown cells. Dependence of transport functions on lipid composition also has been shown for bacteria (33) and yeasts (34).

In conclusion, this study demonstrates that growth conditions have a marked effect on the levels of phospholipids as well as their submolecular species, which in turn influence the transport functions of *M. gypseum.*

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Incorporation of *trans* Fatty Acids into Submandibular Salivary Gland Lipids

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ABSTRACT

Three groups of rats were fed diets containing 20% corn oil, 20% margarine stock (MS) or 19% MS + 1% corn oil. Diets were fed for 12 weeks, 1 week of pregnancy, 3 weeks of lactation and 8 weeks post-weaning. The incorporation of *trans*-octadecenoate into various lipids of the submandibular salivary gland (SMSG) homogenates and plasma membranes was studied. *Trans* octadecenoate was incorporated into all the lipid fractions studied. Its levels were the highest in phosphatidylethanolamine. The double bond index of phospholipid fatty acids in the plasma membranes of the SMSG was substantially lower in the group fed 20% MS. The fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) was generally higher in the membranes of SMSG from rats fed MS than that of the other two groups, thus indicating lower fluidity. Also, the breakpoints in fluorescence polarization were at a higher temperature in the membranes from rats fed MS as compared with those fed corn oil. Lower fluidity of plasma membranes of SMSG observed in rats fed 20% MS may result in modification of the activities of membrane-bound enzymes.
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INTRODUCTION

The consumption of fats of vegetable origin has more than tripled between 1909-13 and 1980 (1). Partially hydrogenated vegetable oils contain large amounts of positional and geometric isomers of fatty acids. These fatty acids are known to be incorporated into the tissues of experimental animals and man fed diets containing partially hydrogenated oils. The extent of their incorporation depends upon the type of isomer, dietary level, length of the feeding study and the type of tissue and lipid being analyzed. Studies concerning the incorporation of *trans* fatty acids in animal tissues (2) and man (3), their metabolism (4,5), biological (6) and nutritional effects (7-9) have been reviewed. Most of the evidence indicates that *trans* fatty acids which occur most commonly in foods do not have any adverse effects provided the diet contains adequate amounts of the essential fatty acids (6,8).

The mechanism whereby the type and the level of the dietary fats can affect the structure and function of the salivary glands has been studied in our laboratory. Previous investigations have shown that the level of dietary fat (10) and essential fatty acid deficiency (11,12) have a profound influence on the fatty acid composition of SMSG lipids. These changes in fatty acid composition were associated with altered gland function (11) and increased $(Na^+ + K^+)$-ATPase activity (12).

In the present study we report the effects of feeding diets containing margarine stock which has high levels of *trans* octadecenoate on the fatty acid composition of homogenates and plasma membranes of SMSG. Since the fluidity of membranes is considered to be important in regulating a number of physiological processes including cellular transport, we also investigated the effects of feeding *trans* fatty acids on membrane fluidity using the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) as a probe.

MATERIALS AND METHODS

Casein (vitamin-free) was purchased from TEKLAD (Madison, Wisconsin). The remaining dietary ingredients were purchased from ICN (Cleveland, Ohio). Margarine stock was supplied by Kraft Inc. (Glenview, Illinois). Corn oil and sucrose were purchased from a local supermarket.

All organic solvents were of analytical reagent grade and were glass-distilled prior to use. Biochemical reagents were purchased from Sigma Chemical Co. (St. Louis, Missouri). Standards for fatty acid methyl esters, including *trans* 16:1 and *cis* 20:1, were obtained from Applied Science (Deerfield, Illinois). Purity of the standards was greater than 99% by GC analyses. Silicic acid (Biosil-A) used for column chromatography was a product of Bio-Rad Laboratories

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[1]Part of this work was presented at the Federation of American Societies for Experimental Biology (FASEB) 68th Annual Meeting, St. Louis, Missouri, April 1984. Alam, S.Q., Alam, B.S., and Banerji, A. Fed. proc. 43,317 (1984).

(Richmond, California).

Animal Study

Fifteen-day pregnant, Sprague-Dawley rats (Holtzman Co., Madison, Wisconsin) were divided into 3 groups of 3-4 rats each and were fed ad libitum semipurified diets containing 20% corn oil (CO), Group I, control; 20% margarine stock (MS), Group II, experimental, or 19% MS + 1% CO, Group III, experimental. The basal diet contained (in weight per cent): casein, 20.0; sucrose, 50.8; cellulose, 4.0; fat or oil, 20.0; mineral mixture (AIN-76), 4.0; vitamin mixture (AIN-76), 1.0; choline chloride, 0.2, and butylated hydroxytoluene, 0.002%, added as an antioxidant. Diets were prepared fresh every 2-3 weeks and were stored at 4 C.

Within 24 hr after delivery, the pups within each dietary group were randomized so there were 8-9 in each foster-litter. Throughout lactation the dams continued feeding on the same respective diets. The pups were weaned at 3 weeks, housed individually in wire-bottomed galvanized cages and fed ad libitum the same diets previously fed to their mothers. Rats were weighed once a week and the food intake was measured 3-4 times during the study. After a total of 12 weeks on their respective diets (1 week of pregnancy, 3 weeks of lactation and 8 weeks post-weaning), rats from each group were decapitated and SMSG were dissected out, rinsed with physiological saline and weighed. Glands from 5-6 rats within each group were pooled (3-4 g wet weight) and minced finely with scissors. Homogenates were prepared in a Potter-Elvejhem tissue homogenizer by homogenizing with 20-25 strokes at 3000 rev/min in 9 volumes (w/v) of a medium containing 0.32 M sucrose in 0.05 M Tris-HCl, pH 7.4 (at 25 C), 0.025 M KCl, 0.003 M $MgCl_2$ and 0.002 M $CaCl_2$. The homogenates were filtered through 4 layers of cheesecloth to remove the connective tissues. The filtered homogenates were used for the preparation of plasma membranes by the differential centrifugation method of Durham et al. (13).

Analytical Procedures

Aliquots of plasma membranes and tissue homogenates were used for lipid extraction using Bligh and Dyer's method (14). The lipid extracts were diluted to a volume of 5 ml with chloroform, and portions were used for the fatty acid determination after transesterification with boron trifluoride-methanol (15). Aliquots of total lipid extracts were separated by silicic acid column chromatography (1 g Biosil-A, 100-200 mesh in a 1 cm, ID column) to remove the phospholipid fraction from neutral lipids and glycolipids (16). Total cholesterol was determined after saponification of the total lipid extract as previously described (17). Stigmasterol was added as an internal standard prior to saponification. Lipid phosphorus was determined by Bartlett's procedures (18). The composition of fatty acid methyl esters of total lipids and phospholipids was determined by gas-liquid chromatography on a Perkin-Elmer 900 gas chromatograph with FID using a glass column (3.8 m × 2 mm) packed with 15% Silar-10C on Gas-Chrom Q 100/200 mesh (Applied Science, Deerfield, Illinois). The column temperature was 230 C, the detector and injection port temperatures were 275 C. Under these experimental conditions t-16:1 and c-16:1 isomers were completely resolved, whereas methyl oleate and methyl elaidate were not. The latter eluted prior to methyl oleate as a shoulder. Since *cis-trans* isomers of 18:1 could not be completely resolved using this column, argentation thin layer chromatography (TLC) was used for this purpose. Glass plates (30 cm × 20 cm, 0.25 mm thick) were prepared by spreading a slurry containing 30 g of silica gel G, mixed with 60 ml of water containing 2.55 g of $AgNO_3$ and 0.1 ml of dichlorofluoroscein (0.2% methanol solution). After drying at room temperature, the plates were activated at 110 C for 1 hr. For the quantitation of *trans* and *cis* isomers of 18:1, internal standards of t-16:1 and c-20:1 (25 μ g each) were added to each sample containing methyl esters of total lipids or phospholipids, and these were spotted on the TLC plates along with the internal standards. The plates were developed in the dark in the 30-cm direction using chloroform-ethanol (99.25:0.75, v/v). Bands were visualized under ultraviolet light. The fluorescent spots containing *cis* and *trans* isomers of monoenoic fatty acid methyl esters were scraped off the plates and extracted twice with methylene chloride-1N anhydrous methanolic HCl (99:1) to elute the esters from the silica gel (19). The solvents were evaporated under nitrogen, the residue was dissolved in 50 μ l of hexane, and aliquots were injected into the gas chromatographic column as described above for the separation of fatty acid methyl esters. The *cis* and *trans* isomers of 18:1 were quantitated by comparison with the internal standards, t-16:1 for t-18:1 and c-20:1 for c-18:1. The values were corrected for slight differences in the detector response for methyl esters of t-16:1 and c-20:1 and for the endogenous levels of t-16:1. The latter were determined from the GLC analyses prior to the addition of the internal standard and constituted 0.7-1.5% of the

TABLE 1

Dietary Fatty Acid Composition

Fatty acid	Diet fed 20% CO	20% MS	19% MS + 1% CO
16:0	10.5	10.2	10.1
18:0	2.3	10.2	10.4
t-18:1	–	51.3	49.4
c-18:1	25.9	22.7	20.8
c,t-18:2*	–	2.0	2.1
c,c-18:2	60.4	1.9	5.8
18:3	0.7	–	–

Values are area per cent, average of 2 determinations.

* includes some t,c and t,t.

CO = corn oil, MS = margarine stock.

total fatty acids.

Duplicate samples of the diets also were extracted for total lipids, and the fatty acid composition was determined using argentation TLC and gas chromatography as described above. The data are presented in Table 1. *Trans* octadecenoate constituted about 50% of the total fatty acids in the two diets containing MS. About 2% of octadecadienoate (t,t plus t,c plus c,t) was also present in the MS diets. The levels of linoleic acid (expressed as % of the total fatty acids) were 1.9 in the 20% MS diet, 5.8 in the 19% MS + 1% CO diet, and 60.4 in the 20% CO diet.

Fluorescence Polarization Studies with Plasma Membranes from SMSG

Fluorescence polarization was measured according to the procedures of Shinitzky and Inbar (20) and Shinitzky and Barenholz (21). A solution of 2×10^{-3} M diphenyl hexatriene (DPH) in tetrahydrofuran was diluted 1000 fold by injection into vigorously stirred phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 12.2 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, pH 7.2). The resulting DPH solution (2×10^{-6} M) was mixed 1:1 (v/v) with the membranes (about 0.5 mg protein), and incubated at 37 C for 1 hr. The incorporation of DPH into the membrane was followed by a steep increase in fluorescence intensity, measured on a SLM 4800 fluorescence polarization spectrophotometer (SLM Instruments, Inc.) with two cross polarized channels. The Excitation λ was 366 nm. Scattering of the emitted light was reduced by using a 389 nm cut off filter. Fluorescence polarization was measured at different temperatures using a water circulating bath (NESLAB Model RTE-9, Portsmouth, New Hampshire). The results are the average of two determinations on a pooled sample within each group. The duplicate values varied from 5-10%.

RESULTS

There was no significant difference in weight gains of rats among the three dietary groups. The concentrations of total lipids, proteins, phospholipids, cholesterol and cholesterol to phospholipid ratios in the SMSG are shown in Table 2. There was no significant difference in any of these parameters except in phospholipid phosphorus, which was higher in the group fed 20% MS when compared with the control group.

The fatty acid composition of total lipids in the SMSG of rats fed the three diets is shown in Table 3. *Trans* 18:1 constituted 17.8% of the total fatty acids in the group fed the 20% MS and 13.9% in the group fed the 19% MS + 1% CO. This difference was significant ($P < 0.05$). There was also some incorporation of c,t-18:2 (plus t,c plus t,t) in the total lipids of the SMSG of the two groups fed MS. A small amount (less than 1%) of the t-16:1 fatty acid was also present.

TABLE 2

Total Lipids, Phospholipids and Cholesterol Content in SMSG of Rats Fed Diets Containing *Trans* Fatty Acids

Dietary group	Total lipids[a] (%)	Proteins[a] (%)	Phospholipid P (nmol/mg protein)	Total cholesterol (nmol/mg protein)	Cholesterol / Phospholipid
20% CO	2.84 ± 0.33	13.05 ± 0.61	165.7 ± 10.7	59.0 ± 4.4	0.356 ± 0.014
20% MS	2.41 ± 0.12	12.21 ± 0.79	205.7[b] ± 11.6	66.1 ± 3.6	0.321 ± 0.008
19% MS + 1% CO	2.88 ± 0.22	12.01 ± 0.53	191.3 ± 11.3	68.5 ± 6.1	0.358 ± 0.021

Values are mean ± SE (5 rats/group).

[a]Expressed as % of the wet weight of the gland.

[b]Significantly different from the control group (20% CO), $P < 0.05$.

CO = corn oil, MS = margarine stock.

TABLE 3

Fatty Acid Composition of Total Lipids of SMSG of Rats Fed Diets Containing *Trans* Fatty Acids

Fatty acid	20% CO	Diet fed 20% MS	19% MS + 1% CO
14:0	1.0 ± 0.14[a]	1.0 ± 0.05[a]	1.2 ± 0.15[a]
16:0	26.6 ± 1.22[a]	17.4 ± 0.60[b]	20.9 ± 0.31[d]
t-16:1	–[a]	0.9 ± 0.09[b]	0.7 ± 0.07[b]
c-16:1	1.3 ± 0.13[a]	4.6 ± 0.12[b]	2.3 ± 0.27[d]
18:0	15.6 ± 0.73[a]	7.6 ± 0.24[b]	9.9 ± 0.17[d]
t-18:1	–[a]	17.8 ± 0.82[b]	13.9 ± 0.64[d]
c-18:1	14.6 ± 0.63[a]	34.8 ± 0.78[b]	20.9 ± 0.53[d]
t,c-18:2*	–[a]	2.9 ± 0.12[b]	1.3 ± 0.17[d]
c,c-18:2	21.0 ± 1.05[a]	2.8 ± 0.57[b]	9.6 ± 0.37[d]
20:2	1.0 ± 0.17[a]	1.3 ± 0.18[a]	0.4 ± 0[b]
20:3 ω9	–[a]	3.6 ± 0.65[b]	0.6 ± 0.06[a,d]
20:3 ω6	3.1 ± 0.16[a]	1.3 ± 0.28[b]	2.0 ± 0.31[b]
20:4	15.0 ± 1.18[a]	3.2 ± 0.80[b]	11.5 ± 0.54[d]

Values are area per cent (mean ± SE, 5 rats/group).

Means within the same line with a superscript letter in common are not significantly different. Means with different superscripts are significantly different ($P < 0.05$), using analysis of variance, Neumann-Keul's *test*.

c designates fatty acid containing *cis* double bond.

t designates fatty acid containing a *trans* double bond.

* or c,t or t,t. – indicates trace.

CO = corn oil, MS = margarine stock.

TABLE 4

Fatty Acid Composition of Neutral Lipids, Free Fatty Acids and Triglycerides of SMSG of Rats Fed Diets Containing *Trans* Fatty Acids

Group no.	14:0	16:0	t-16:1	c-16:1	18:0	t-18:1	c-18:1	t,c-18:2*	c,c-18:2	20:3 ω9	20:3 ω6	20:4
Neutral lipids												
I	1.6	25.1	–	2.9	11.1	–	17.3	–	25.0	–	1.4	9.9
II	1.9	18.1	1.4	6.6	5.3	21.8	29.5	3.1	3.0	2.9	–	3.2
III	1.7	18.0	1.1	3.7	7.2	13.1	23.3	1.7	8.0	0.7	2.1	15.3
Free fatty acids												
I	1.3	26.0	–	1.6	14.8	–	14.9	–	22.8	–	1.9	12.2
II	1.1	12.3	1.3	5.1	6.2	17.9	31.2	2.8	3.8	5.7	–	5.1
III	1.2	16.4	1.1	3.3	9.2	13.6	23.1	1.7	9.9	0.7	2.1	15.2
Triglycerides												
I	2.9	30.5	1.3	4.7	4.6	–	25.3	–	25.9	–	–	0.8
II	2.7	26.7	1.5	8.2	3.9	17.4	33.6	2.3	1.2	2.5	–	–
III	2.5	26.7	1.4	5.8	5.4	20.0	29.8	2.4	4.0	1.0	–	0.9

Values are area per cent of pooled samples within each dietary group.

c designates fatty acid containing *cis* double bond; t designates fatty acid containing a *trans* double bond.

* or c,t or t,t.

– indicates trace.

I = 20% CO; II = 20% MS; III = 19% MS + 1% CO.

The fatty acid composition of total lipids in the SMSG of rats fed 20% MS diet was similar to that observed in essential fatty acids (EFA) deficiency. An increase in the levels of c-16:1 and c-18:1, a decrease in c,c18:2 and 20:4 and an increase in 20:3ω9 were observed. The addition of 1% corn oil to the diet tended to restore the normal fatty acid patterns of total lipids in the SMSG.

The fatty acid composition of neutral lipids, free fatty acids and triglycerides in the SMSG of rats fed the three diets is shown in Table 4. The patterns typical of EFA deficiency such as an increase in c-16:1, c-18:1 and 20:3ω9, and a decrease in c,c-18:2 and 20:4, were observed in rats fed the 20% MS diet. The incorporation of

TABLE 5

Fatty Acid Composition of Phospholipids, Phosphatidylcholine and Phosphatidylethanolamine of SMSG of Rats Fed Diets Containing *Trans* Fatty Acids

Group no.	14:0	16:0	c-16:1	18:0	t-18:1	c-181	t,c-18:2*	c,c-18:2	20:3 ω9	20:3 ω6	20:4
Phospholipids											
I	0.9	25.2	3.4	15.5	–	11.1	–	18.3	–	3.4	16.1
II	0.8	16.1	6.5	7.6	13.6	36.7	–	5.1	5.0	–	3.9
III	1.3	21.2	5.2	9.9	8.0	23.7	2.8	11.1	0.7	1.7	10.7
Phosphatidyl-choline											
I	2.8	38.7	2.0	23.7	–	11.0	–	10.0	–	3.3	4.9
II	3.2	23.7	2.7	10.8	11.1	34.5	1.8	6.6	2.6	1.3	0.6
III	3.5	33.4	0.5	13.2	12.0	18.1	–	5.2	6.2	2.3	3.0
Phosphatidyl-ethanolamine											
I	2.2	14.7	3.5	28.9	–	21.0	–	11.4	–	2.4	9.6
II	1.1	7.4	6.0	10.3	24.8	25.3	6.9	5.0	3.5	–	1.8
III	1.8	10.7	4.2	15.1	20.3	20.8	4.5	6.6	1.7	–	6.3

Values are area per cent of pooled samples within each dietary group.
c designates fatty acids containing *cis* double bonds; t designates fatty acids containing a *trans* double bond.
* or c,t or t,t.
– indicates trace.

TABLE 6

Fatty Acid Composition of Total Lipids of SMSG Plasma Membranes of Rats Fed Diets Containing *Trans* Fatty Acids

Fatty acid	20% CO	Diet fed 20% MS	19% MS + 1% CO
14:0	2.3 ± 1.0[a]	2.2 ± 0.8[a]	1.9 ± 0.9[a]
16:0	27.6 ± 1.0[a]	16.4 ± 0.1[b]	18.1 ± 0.5[b]
t-16:1	–[a]	0.8 ± 0.1[b]	1.4 ± 0.3[b]
c-16:1	1.7 ± 0.3[a]	3.7 ± 0.3[a]	2.3 ± 0.7[a]
18:0	15.4 ± 0.8[a]	9.3 ± 0.7[b]	11.2 ± 0.5[b]
t-18:1	–[a]	11.6 ± 0.3[b]	11.4 ± 0.8[b]
c-18:1	18.0 ± 2.9[a]	39.5 ± 2.6[b]	23.8 ± 2.5[a]
t,c-18:2*	–[a]	3.3 ± 0.4[a]	1.8 ± 1.25[a]
c,c-18:2	14.2 ± 0.4[a]	2.3 ± 0.2[b]	8.3 ± 1.0[d]
20:3 ω9	–[a]	4.4 ± 0.7[b]	0.8 ± 0.3[a]
20:3 ω6	2.8 ± 0.6[a]	–[b]	1.9 ± 0.3[a]
20:4	14.2 ± 2.0[a]	2.6 ± 0.4[b]	13.7 ± 0.4[a]

Values are mean ± SEM of 3 preparations obtained from 15-18 rats per group.
Means within the same line with a superscript letter in common are not significantly different $P < 0.05$. Means with different superscripts are significantly different, using analysis of variance, Neumann-Keul's test.
c designates fatty acid containing *cis* double bond; t designates fatty acid containing a *trans* double bond.
* or c,t or t,t.
– indicates trace.
CO = corn oil, MS = margarine stock.

1% corn oil in this diet at the expense of the same amount of MS resulted in a trend toward restoration of the normal fatty acid patterns. *Trans* octadecenoate was present at a level of 13.1% to 21.8% in the neutral lipids, triglycerides and free fatty acid fractions of SMSG of Groups II and III of rats fed MS. Small amounts of t-16:1 and t-18:2 (t,c plus c,t plus t,t) were also present in these two experimental groups. The fatty acid composition of cholesterol esters was not determined.

Data on the fatty acid patterns of phospholipids, phosphatidylcholine and phosphatidylethanolamine fractions are shown in Table 5. Patterns typical of EFA deficiency also were observed in these phospholipid fractions of SMSG of rats fed 20% MS. The inclusion of 1% corn oil in the diet (Group III) resulted in a trend toward restoration of the normal fatty acid patterns. *Trans* 18:1 was present in the two groups fed MS. Its levels were the highest in the phosphatidylethanolamine fraction.

TABLE 7

Fatty Acid Composition of Phospholipids of SMSG Plasma Membranes of Rats Fed Diets Containing *Trans* Fatty Acids

Fatty acid	Diet fed 20% CO	20% MS	19% MS + 1% CO
14:0	4.6	3.3	4.1
16:0	26.7	17.1	21.3
t-16:1	–	–	1.8
c-16:1	1.8	5.2	2.3
18:0	16.6	9.0	11.2
t-18:1	–	10.3	8.1
c-18:1	12.0	41.1	27.6
c,c-18:2	16.7	2.8	9.1
20:3 ω9	–	4.0	0.7
20:3 ω6	3.2	0.1	1.7
20:4	16.1	3.2	12.1
Double bond index	121.2	77.0 (87.3)	103.7 (113.6)

Values are area per cent of pooled sample of 3 preparations obtained from 15-18 rats per group.
( ) designates double bond index with t-16:1 and t-18:1 included as unsaturated fatty acids.

The fatty acid composition of total lipids and phospholipids in plasma membranes from the SMSG of rats fed the three diets is shown in Tables 6 and 7, respectively. Similar patterns were observed in plasma membrane lipids as in the gland homogenates. The effects of feeding diets containing MS on the fatty acid composition and the incorporation of t-18:1 fatty acid were similar to those observed in the gland homogenates. The double bond index of fatty acids defined as a sum of (% fatty acid)×(number of double bonds) was the lowest in plasma membrane phospholipids of rats fed 20% MS and was intermediate in the group fed the diet with 19% MS + 1% CO.

The fluorescence polarization of DPH measured at different temperatures in pooled samples of plasma membranes of the SMSG pertaining to the three dietary groups is shown in Figure 1. Throughout the entire temperature range (5 to 40 C), the fluorescence polarization of DPH was lower in the membranes of the control group (20% CO) than in those of the two experimental groups. The highest values were obtained for the membranes prepared from the SMSG of rats fed 20% MS; the values for the group fed 19% MS + 1% CO were between the other two groups. Similarly, the break points (defined as the point indicating a sudden change in slope) in fluorescence polarization of DPH were at lower temperatures in the control group than in the 20% MS group (22 C and 12 C in the control group versus 32 C and 20 C in the 20% MS group). The break points in fluorescence polarization in the membranes of the 19% MS + 1% CO were between the other two groups.

DISCUSSION

The results of the present study show that dietary *trans* octadecenoate was incorporated into the total lipids, phospholipids and neutral lipids of the SMSG. The level of its incorporation depended upon the type of lipid; maximum incorporation was in the phosphatidylethanolamine fraction. It constituted about 25% of the total fatty acids in this lipid fraction. Other investigators have reported similar findings with several other tissues (2,22-24). The plasma membranes isolated from the SMSG also contained 11-12% of t-18:1 in the total lipids and phospholipids of rats fed MS.

In addition to the incorporation of t-18:1 into various lipids, changes typical of EFA deficiency were observed in the fatty acid profiles of rats fed 20% MS. These changes consisted of higher levels of c-16:1, c-18:1 and lower levels of c,c-18:2 and 20:4. The levels of 20:3 ω9 also were increased. When 1% margarine stock was replaced by corn oil in the diet, these changes in fatty acid patterns were almost alleviated. In fact, the ratio of 20:3 ω9 to 20:4 in total lipids of the SMSG was decreased from 1.1 in the 20% MS group to 0.05 in the group fed 19% MS + 1% CO. A ratio less than 0.4 is considered to be adequate in terms of EFA requirements (25). Rats in Group III (19% MS + 1% CO) are much more akin to practical human nutrition since their diet is sufficient in EFA. Therefore, the observed changes can be attributed only to the *trans* fatty acids and are not confounded by a concomitant EFA deficiency.

The presence of small amounts of *trans* hexadecenoate in lipids of SMSG indicates that it may be synthesized by the tissues since it is not present in the diet. There is some evidence that *trans* hexadecenoate may be produced by chain-shortening or retroconversion from *trans* octadecenoate (26). Similarly, *trans, cis*-octadecadienoate (or *cis, trans*) may be produced from *trans* octadecenoate by desaturation. Small amounts of these octadecadienoate isomers were also present in the two diets containing MS.

Fluorescence polarization of DPH showed that the lipid environment in the plasma membranes of SMSG from rats fed *trans* fatty acids was less fluid than that of the control group. In addition, the transition temperature was higher in membranes of rats fed MS than that of the control rats. These results are consistent with our findings on the double bond index

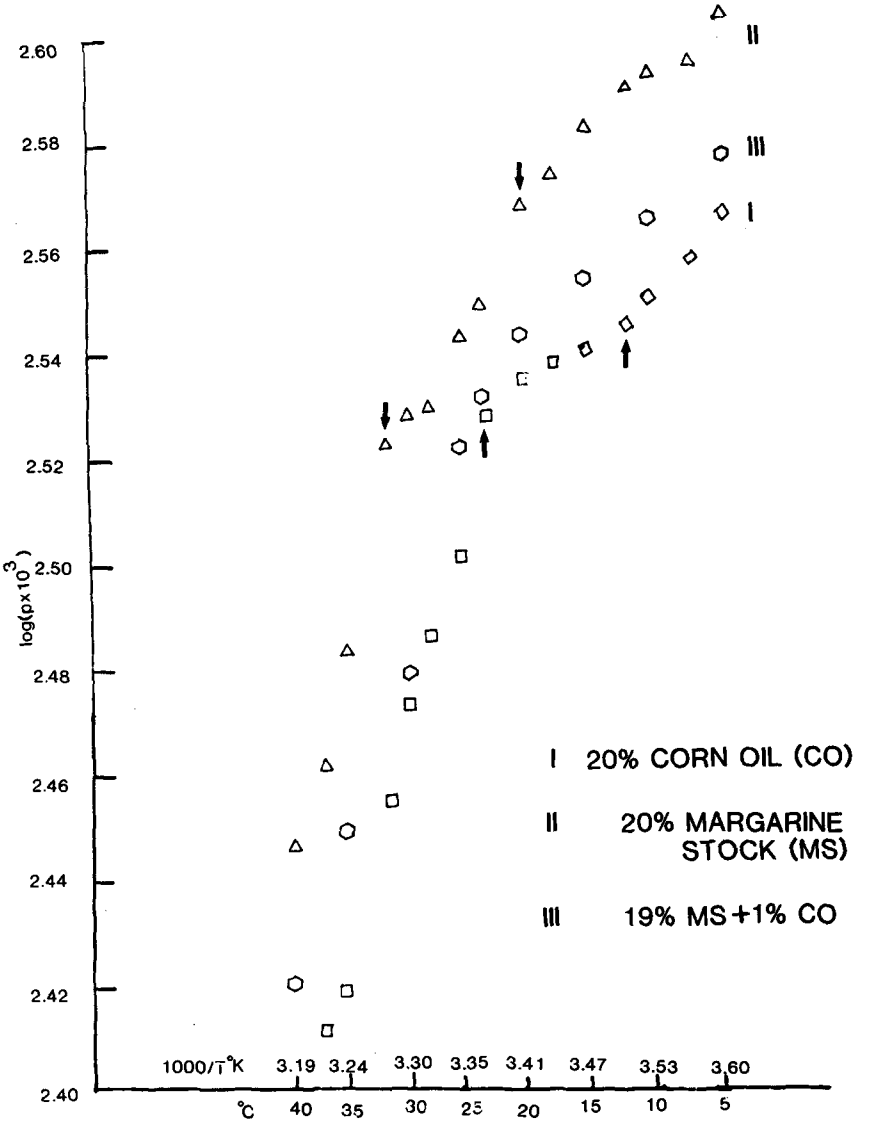

FIG. 1. Fluorescence polarization of DPH in plasma membranes from SMSG of rats fed diets containing *trans* fatty acids. Group I, 20% CO (control); Group II, 20% MS (experimental); Group III, 19% MS + 1% CO (experimental). Fluorescence polarization was measured at different temperatures (5-40 C) as described under Materials and Methods. Arrows indicate the breakpoints where a sudden change in slope takes place.

which also showed less unsaturation and therefore less fluidity in membrane lipids of the SMSG of rats fed MS. Englehard et al. (27), using DPH as a probe, also have reported lower fluidity in plasma membranes of mouse fibroblast cells grown in the presence of *trans* fatty acids, a finding similar to ours. However, our results differ somewhat from those recently reported by Mahfouz et al. (28) on liver microsomal phospholipid membrane vesicles prepared from rats fed diets containing 80% partially hydrogenated soybean oil + 20% corn oil. They did not observe any difference in fluorescence polarization in any of the dietary groups. However, the data from their study is not strictly comparable with ours since we had an additional experimental group fed diet containing *trans* fatty acids without any corn oil (Group II, 20% MS). It is the membranes from this group which showed markedly higher fluorescence polariza-

tion values compared with the control group fed 20% corn oil. Fluorescence polarization values in membranes prepared from rats fed 19% MS + 1% corn oil (Group III), a group more comparable with that of Mahfouz et al. (28), tended to be closer to those of the control group. The use of different probes (diphenylhexatriene versus *trans*-parinaric acid) and different systems (plasma membranes versus phospholipid vesicles) may be additional explanations for the observed difference between the two studies. As compared with phospholipid vesicles, plasma membranes represent a more complex biological system involving not only lipid-lipid, but also lipid-protein interactions.

The question, are these effects of *trans* fatty acids on SMSG plasma membrane lipid composition and fluidity associated with changes in the activities of membrane-associated enzymes such as $(Na + K^+)$-ATPase and adenylate cyclase, is being investigated currently in our laboratory.

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The Interaction of Human Plasma Low Density Lipoproteins with Glycosamino-glycans: Influence of the Chemical Composition

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ABSTRACT

Human plasma of 5 normolipemic individuals was incubated for 24 hr at 37 C in the presence or in the absence of lecithin:cholesterol acyltransferase (LCAT)-inhibitors. Plasma stored at 4 C served as a control. The low density lipoprotein (LDL) fractions of the samples were isolated and investigated with respect to changes in chemical composition and complexing activity with glycosamino glycans (GAG).

Incubation of plasma in the presence of LCAT inhibitors caused a significant increase of LDL triglycerides at the expense of cholesteryl esters. Incubation with active LCAT not only changed the core but also the surface constituents (decrease in phospholipids and in free cholesterol).

The amount of GAG bound per mg of LDL was not uniformly changed in samples incubated after LCAT inhibition. LDL isolated from plasma incubated in the presence of LCAT, on the other hand, showed a significant reduction in GAG binding.

The ratio of free cholesterol:GAG in the complex was most significantly reduced in LCAT-modified LDL. There was in addition a highly significant correlation between the LDL:GAG ratio in the complex and the free cholesterol and phospholipid content of the LDL samples.

It is concluded that alterations in surface lipid constituents of LDL strongly affect their interaction with sulfated polysaccharides, an effect which may be relevant also in vivo for the interaction of LDL with cell surfaces and intercellular matrices.
Lipids 20:24-28, 1985.

INTRODUCTION

Low density lipoproteins (LDL), the major cholesterol and cholesteryl ester transporting fraction in human plasma, play a dominant role in atherogenesis; there exists a great body of evidence indicating that plasma LDL and LDL-cholesterol concentrations correlate significantly with the incidence of coronary artery diseases and myocardial infarction (1-3). The pathophysiological events involved have not been clarified in all details, and numerous theories exist which may help in part to understand this problem. One of these theories relates to the interaction of LDL with various kinds of sulfated polysaccharides of the heparin-, chondroitin sulfate- and heparan sulfate type. Such polysaccharides may play a role as low affinity receptors for apoB-containing lipoproteins which are abundant on cell surfaces of many organs (4). Sulfated polysaccharides covalently linked to proteins, on the other hand, are the most important substances forming the intercellular matrix of fibroblasts and smooth muscle cells. The known interaction of LDL with such glycosamino glycans (GAG) in the arteries most likely is a key feature in the events leading to cholesterol deposition in atherosclerotic plaques (for a review see 5).

The interaction of apoB-containing lipoproteins with GAG has been studied intensively in many laboratories including our own (6-9) and is known to be influenced by many factors. In this study we investigated the lipoprotein complexing activity of GAG with freshly isolated LDL in comparison to LDL which had been chemically modified after incubation with lecithin:cholesterol acyltransferase (LCAT)-active plasma.

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MATERIALS AND METHODS

For preparations of LDL, blood samples were obtained from healthy normolipemic male and female volunteers. After the addition of 1.5 mg/ml of Na_2EDTA, plasma was prepared by low speed centrifugation. The plasma was divided into three equal parts and treated as follows: Sample 1 was stored at 4 C for 24 hr with 5 mmol/l of Na-iodoacetate. Sample 2 was incubated for 24 hr at 37 C in the dark and under nitrogen plus 5 mmol/l of Na-iodoacetate. Sample 3 was incubated the same way as Sample 2, but without adding any LCAT inhibitor. In addition, all samples contained streptomycin (50 μg/ml) and penicillin (50 IU/ml) to avoid microbial growth.

In control experiments these antibiotics were omitted, and other LCAT inhibitors, e.g. diisopropyl fluorphosphate or Ellman's reagent, were added to the plasma without any noticeable changes in the results. After incubation

5 mmol/l of Na-iodoacetate were added to Sample 3 and the density of all plasma samples was adjusted to 1.020 g/ml by adding solid NaCl. VLDL plus IDL were separated by ultracentrifugation for 20 hr at 42.000 rpm and 15 C by using the 60-Ti rotor (Beckman). After separation of the top fraction by tube slicing, the density of the infranatant was adjusted to 1.070 g/ml with NaCl followed by a second spin in the ultracentrifuge (45.000 rpm, 15 C, 24 hr). The floating LDL were further purified in a linear gradient, d:1.020-1.070 g/ml, using the SW 41 rotor from Beckmann (41.000 rpm, 15 C, 24 hr). LDL which concentrated as a homogenous band in the middle of the tube was collected by aspiration and dialyzed against 0.15 M NaCl, 10 mM Tris. HCl, pH 7.4 and 1 mg/ml of EDTA and NaN respectively. The LDLs isolated from samples 1-3 will be referred to as "LDL-4," "LDL-37" and "LDL-37 + LCAT."

The chemical composition of lipoprotein fractions was determined by standard methods as described earlier (10). Protein was determined according to Lowry, using human serum albumin as a standard; free and esterified cholesterol were assayed by the esterase/oxydase kit from E. Merck (Darmstadt); phospholipids were determined enzymatically with the kit from Biomerieux (France), and triglycerides with the kit from Böhringer (Mannheim, Germany). All chemicals were reagent grade and from E. Merck, Darmstadt.

The hydrated densities of LDLs were determined by density measurements in a precision densitometer DMA 60 (Anton Paar, Graz) as outlined in detail earlier (11). LDL concentrations were determined gravimetrically after equilibrium analysis and evaporation in a vacuum desiccator.

Electron microscopy. Negative stain EM was performed on a Phillips EM 300 using a 2% solution of Na-phosphotungstate. Photographs were taken at a magnification of 68.000 and the mean particle diameters were evaluated from measuring of 300 particles per sample.

Measurement of the LDL-GAG interaction. GAG was prepared according to a modification of our previous procedure (7). Human aortic tissues were delipidated with diethyl ether followed by proteolytic digestion for 24 hr at 65 C (2 mg papain, 6 mg EDTA, 2.7 mg cysteine. HCl in 2 ml of 0.1 M phosphate buffer, pH 6.4). The samples were deproteinized by passing through a DOWEX 50W-X2 column. GAG's were identified by a combination of electrophoresis, susceptibility to digestion with specific enzymes and reaction with HNO_2. The preparations consisted of 80% chondroitin sulfate-dermatan sulfate, 17% heparan sulfate and 3% hyaluronic acid. The application of mixtures of 80% chondroitin sulfate standard and 20% heparan sulfate standard in a control experiment gave very similar results. Prior to the start of these experiments samples were dialyzed for 12 hr at 4 C against 0.15 M NaCl. The ratio of GAG to LDL was estimated by titrating fixed amounts of GAG with increasing amounts of LDL and by reverse titration of LDL with GAG as described previously (9). The recovery of LDL in the insoluble complex was determined by protein analysis. GAG concentrations were measured according to Bitter and Muir (12). GAG reference standards were a generous gift of M.B. Mathens and J.A. Cifonelli (Chicago, Illinois).

RESULTS

Table 1 lists the chemical composition as well as the hydrated density of the different LDL samples. The numbers represent means ± SD of five consecutive experiments. The incubation of plasma in the presence of active LCAT caused a significant change of all LDL constituents. The most striking differences were noticed in the content of free cholesterol, which fell by almost 50%, and in triglycerides, which rose by 80%. Phospholipids decreased by 18% and the choles-

TABLE 1

Chemical Composition and Physicochemical Properties of the LDL Preparations

Samples	% by weight					$\bar{v}$	Diameter ϕ Å
	Protein	CE	FC	PL	TG		
LDL-4	23.7 ± 0.7	39.3 ± 1.3	10.8 ± 0.4	21.6 ± 1.1	4.1 ± 1.7	1.037 ± 0.002	218± 4
LDL-37	23.9 ± 1.0	37.6 ± 2.0*	9.9 ± 0.3	21.4 ± 1.2	7.2 ± 1.3*	1.033 ± 0.002	222± 5
LDL-37 + LCAT	25.8 ± 1.0*	43.1 ± 1.7*	5.6 ± 0.9*	18.1 ± 0.7*	7.5 ± 1.7*	1.045 ± 0.003*	207± 8*

Values are means ± S.D. of duplicate analysis of 5 different preparations each. The composition is given in % (w/w).

*Significantly different from LDL-4 ($p < 0.001$).

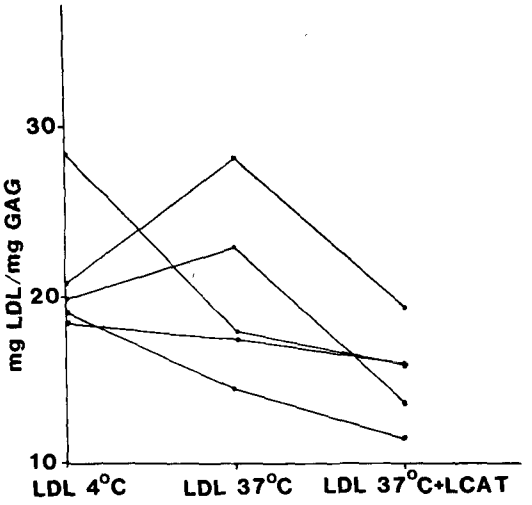

FIG. 1. Binding of LDL samples to GAG: The three different LDL samples, LDL-4, LDL-37 and LDL-37 + LCAT, were titrated with GAG, and the maximal binding in mg LDL bound per mg of GAG is shown.

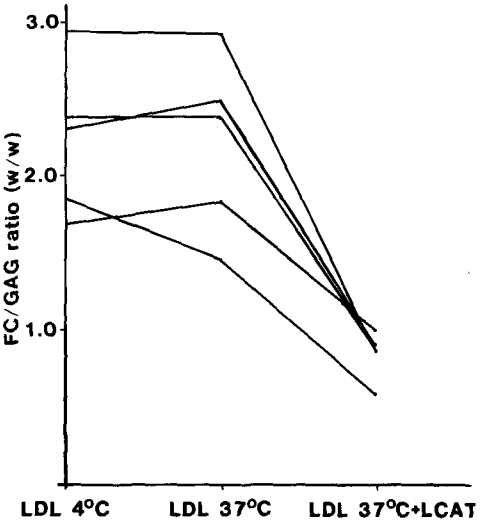

FIG. 2. Plot of the LDL-free cholesterol:GAG ratio (w/w) within the LDL-GAG complexes of the three LDL samples.

teryl ester (CE)- and protein content increased by 8% each. If plasma was incubated in the absence of active LCAT (LDL-37) there was only a significant increase in triglycerides at the expense of CE. The other constituents remained at the level of control LDL (LDL-4). Due to the changes in composition we also noticed alterations in hydrated densities and in particle size (Table 1).

We also have investigated the protein moiety of all three LDL samples by SDS polyacrylamide gel electrophoresis and immunochemically (13). The apoB of LDL remained unchanged during incubation at 37 C with or without LCAT inhibitors, and more than 95% of the LDL protein migrated in a single band as B-100 as noticed already previously (16).

LDL-GAG interaction. In Figure 1 the amount of LDL bound per mg of GAG of the different LDL samples is plotted. Incubation of plasma at 37 C in the absence of LCAT did not result in a uniform change of the amount of LDL bound to GAG. With some samples we

TABLE 2

Weight Ratio of LDL:GAG in the Formed Complexes

Experiment	Samples	LDL/GAG ratio (w/w)	Δ (%)	Δ (%)
1	LDL-4	28.6	56.3	9.7
	LDL-37	17.8		
	LDL-37 + LCAT	16.1		
2	LDL-4	20.4	31.9	39.8
	LDL-37	23.3		
	LDL-37 + LCAT	13.9		
3	LDL-4	18.5	14.6	9.5
	LDL-37	17.5		
	LDL-37 + LCAT	15.8		
4	LDL-4	18.2	35.7	18.9
	LDL-37	14.5		
	LDL-37 + LCAT	11.7		
5	LDL-4	21.7	9.7	31.4
	LDL-37	28.6		
	LDL-37 + LCAT	19.6		

The different LDL preparations were titrated with GAG as described in Materials and Methods.

Values are means of duplicate analysis obtained at maximal binding of GAG.

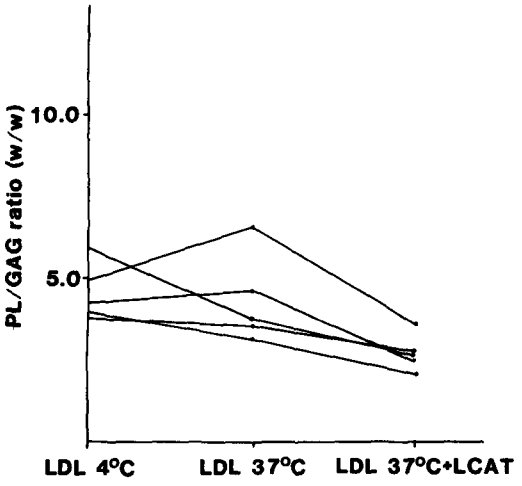

FIG. 3. Plot of the LDL-phospholipid:GAG ratio within the LDL-GAG complexes of the three different LDL samples.

observed an increase, with others a decrease in the amount. If, on the other hand, LDL was isolated from plasma incubated in the presence of LCAT, we noticed in all cases a decrease in binding. The amount of LDL bound decreased by 10-40% as compared to LDL-37 (Table 2). Figures 2 and 3 show the interaction of the different LDL samples expressed in mg of LDL-free cholesterol and in mg of LDL-phospholipids respectively, per mg of GAG. Whereas 4 C and 37 C incubated LDL behaved identically there was a highly significant reduction in LDL binding by GAG observed in the LCAT treated samples.

By plotting the LDL/GAG ratio against the wt. per cent free cholesterol in LDL, we observed a significant positive correlation (Fig. 4). A similar correlation was observed between the LDL/GAG ratio and the wt. per cent phospholipid in LDL (data not shown).

DISCUSSION

During the incubation of freshly drawn human plasma for 24 hr at 37 C there are two main processes which seem to be responsible for the chemical alteration of LDL. The first is caused by the enzyme LCAT leading to the esterification of free cholesterol and the formation of lysolecithin. LCAT has been shown to act primarily on HDL (14), and it is believed that the reaction product which is devoid of surface lipids avidly takes up free cholesterol and phospholipids from other plasma lipoproteins. The second process, which proceeds in parallel, is caused by the exchange and/or transfer of CE (15). CE may exchange against triglycerides mainly from VLDL or may be transferred from HDL to VLDL or to LDL after the LCAT action. All these processes do proceed also in vivo and thus the composition of fasting plasma lipoproteins is in a dynamic equilibrium which is governed by the action of LCAT, exchange/transfer processes and lipases, in addition to the influx and efflux of lipids and lipoproteins from circulation.

In our experiments we tried to force this dynamic to a static equilibrium by incubation of plasma for 24 hr at 37 C. Although we are convinced that the compositional changes of

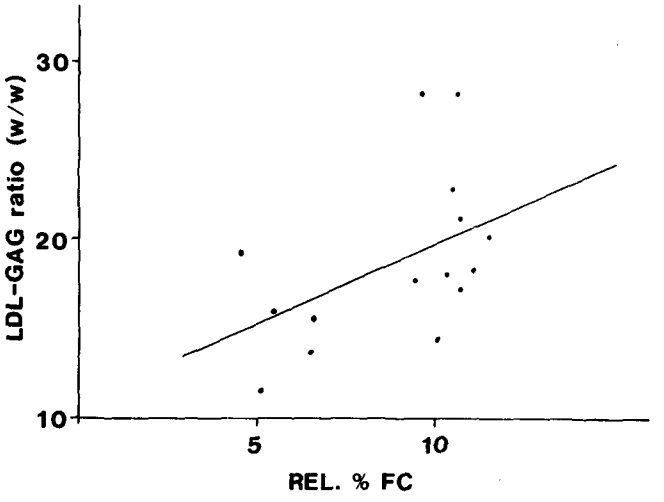

FIG. 4. Plot of the LDL:GAG ratio (w/w) as a function of the relative free cholesterol content of LDLs. This graph contains values of all LDL samples studied irrespective of their pretreatment. The equation of the regression line is: $y = 0.088x + 9.07$; $r = 0.61$; $p < 0.001$.

LDL which we observed were caused predominantly by LCAT in combination with the action of exchange/transfer proteins, we cannot exclude that other processes proceeded in parallel.

In the present investigation, where plasma was incubated at a 5 mM concentration of Na-iodoacetate, LCAT was inhibited by >98% but the exchange activity remained intact. Thus only the content in core lipids was altered. In the absence of any inhibitor, core and surface lipids were modified simultaneously.

In earlier experiments we have demonstrated that the LDL produced in vitro during plasma incubation is chemically and physicochemically altered (13,16). The protein moiety remained unchanged during incubation with regard to apoB, and no fragmentation occurred under these experimental conditions (13). We also have shown that the LCAT-treated LDL exhibited a lower binding affinity to specific cell surface receptors (16). In this study we observed that LCAT treatment also diminished the interaction of LDL with GAG. It has been shown in previous work that phospholipase A treatment of LDL greatly diminishes the interaction with dextran sulfate in the presence of divalent cations (17). In our own investigations, a decreased reactivity between phospholipase-C treated LDL and various GAGs could be demonstrated (18). The modification of LDL by limited tryptic digestion, on the other hand, significantly increased the formation of complexes with GAG. Desialysation did not result in any alteration of the reactivity with LDL (19).

These former enzymes, however, were of non human origin. In this study, human plasma was incubated in its physiological environment and thus the reduction in the phospholipid content of LDL was only 18%. Concommitantly, the relative free cholesterol concentration fell drastically. Due to the action of exchange proteins, the core constituents of LDL also were modified. Whereas this latter modification did not cause any uniform change in the LDL-GAG interaction, we found a highly significant correlation between the content of free cholesterol and of phospholipids with the LDL:GAG ratio in the complex.

Although the LDL-37 + LCAT which we generated must be considered as an artificial product, we believe our findings may also be relevant for the in vivo situation. Under dyslipoproteinemic conditions, the composition of the LDL fraction is shifted toward one extreme or the other (20,21). Such abnormal LDLs may exhibit grossly altered binding characteristics to cell surfaces or toward intercellular matrices and thus may modulate the atherogenicity of that fraction. Such differences in GAG interactions of chemically altered LDL occurring in individuals with hyperlipoproteinemia has been demonstrated by us earlier (9).

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METHODS

A Mild, Rapid, and Efficient Method of Lipid Extraction for Use in Determining Vitamin E/Lipid Ratios

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ABSTRACT

A new, general method for lipid extraction and measurement of vitamin E/total lipid ratios in tissue and cell samples has been developed. The new extraction procedure uses a combination of sodium dodecylsulfate, ethanol and *n*-heptane, and is mild, clean, convenient, efficient and rapid (≤5 min). The efficiency of the new method has been confirmed for human plasma, red blood cells and rat liver homogenate by the comparison of the yields of vitamin E, O-acyl lipid and cholesterol with the yields obtained following conventional extraction procedures. Extraction efficiency also has been confirmed for multilamellar vesicles composed of known quantities of vitamin E, egg lecithin and cholesterol.

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INTRODUCTION

There is increasing interest in the involvement of in vivo lipid peroxidation in the general aging process and in the onset and development of associated diseases, such as heart disease and cancer, with special attention being paid to the effect of certain dietary compounds in retarding these degenerative processes (1,2). Lipid peroxidation, which involves the autoxidation of polyunsaturated fatty acids by a free-radical chain process, can be inhibited dramatically by very small quantities of lipid-soluble chain-breaking antioxidants (3-5). Vitamin E (1) and β-carotene (1,6,7) have received much recent attention with regard to their possible preventive role in these degenerative disease processes. These two compounds are believed to function in vivo as antioxidants, and it has been demonstrated clearly in vitro that each compound is an inhibitor of autoxidation (8,9).

In assessing the susceptibilities of different tissues, cells and organelles to peroxidative damage and the relevance of the findings to degenerative disease processes, it is now evident that lipid-soluble antioxidant levels (e.g., vitamin E, β-carotene) must be measured relative to the peroxidizable lipid (i.e., the polyunsaturated fatty acid residues) found associated with the antioxidants (10). Thus, for example, vitamin E has been reported to vary widely from tissue to tissue (11,12), but only rarely has its concentration been reported relative to total lipid (12,13) or the quantity of polyunsaturated fat (14).

In this paper we report the development of a convenient, new method for extracting lipid from cell and tissue samples which, because of its speed and mildness, greatly facilitates the measurement of small quantities of labile lipid components, such as vitamin E (determined by HPLC), and the measurement of polyunsaturated fatty acid (determined as part of the O-acyl lipid by GC after base-catalyzed transesterification of the lipid extract). The new procedure uses SDS to make membrane lipids amenable to extraction by a combination of ethanol and *n*-heptane. The efficiency of the method has been tested and confirmed by determining the quantities of vitamin E, cholesterol and O-acyl lipid in lipid recovered from aqueous MLV of known composition and also by determining and comparing these same lipid parameters in lipid extracted from red blood cells and rat liver homogenate by both the new procedure and by traditional methods (15-17). Blood plasma also has been used to determine the effect of varying SDS concentrations upon lipid yields by comparing results for lipid recovered by extraction with ethanol/*n*-heptane alone and by extraction with ethanol/*n*-heptane after the addition of SDS.

MATERIALS AND METHODS

Materials

Solvents were HPLC-grade (Fisher) except *n*-heptane (Fisher Spectranalyzed®) and abso-

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Abbreviations: SDS: sodium dodecyl sulfate; MLV: multilamellar vesicles; RBC: red blood cells; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PS: phosphatidylserine; SP: sphingomyelin; 2-BHA: 2-*t*-butyl-4-hydroxyanisole; PMHC: 2,2,5,7,8-pentamethyl-6-hydroxychroman; CMC: critical micelle concentration.

lute ethanol (reagent grade). *t*-Butyl methyl ether (HPLC-grade) was obtained from Burdick and Jackson. The SDS detergent was obtained from Bio-Rad (electrophoresis grade) and from BDH (specially purified for biochemical work). Egg lecithin was bought from Avanti Polar Lipids (Birmingham, Alabama), and reference samples of PC, PE, PS, SP and cardiolipin were supplied by Mrs. A. Martin (NRC). Other chemicals required for reference and/or identification purposes were cholesterol and cholestane (Sigma); various fatty acid methyl esters and triheptadecanoin (Nu-Chek-Prep Inc., Elysian, Minnesota); 2*R*, 4′*R*, 8′*R*-α- and γ-tocopheral (Eastman); 2-BHA (Eastman, now discontinued); and PMHC (synthesized earlier [8] by a published procedure [18]). TLC was performed on silica gel 60 precoated on glass plates (Merck). Sephadex G-25 (coarse) was obtained from Pharmacia Fine Chemicals.

Fresh whole blood was obtained by venipuncture from a human volunteer (A.W.) and was mixed immediately with the anticoagulant, disodium ethylenediaminetetraacetate. Blood also was obtained from the Red Cross Blood Bank.

Rat liver was obtained from a young, male, adult Sprague-Dawley rat (ca. 200 g) and was homogenized with ca. 3 volumes of water in a Tissue Mizer homogenizer (medium setting; 4 x 15 sec).

All aqueous solutions were prepared using doubly-distilled water.

General Procedure for SDS Extraction of Lipid

A given volume of an SDS solution of known concentration (in the range 0.01-0.80 M) was added and mixed with the aqueous sample of plasma, RBC membrane or liver homogenate. A volume of absolute ethanol, usually equal to the combined aqueous volume, was then added and mixed by shaking or brief vortex-stirring. This causes the protein to precipitate immediately. Next, a known volume of *n*-heptane equal to, or in some cases less than, the volume of added ethanol was added and vigorously mixed by vortex-stirring for 30-60 sec. The aqueous and organic layers were conveniently and rapidly separated by brief centrifugation in a clinical, bench-top centrifuge (1-2 min). A known volume of the organic layer was carefully drawn off with a Pasteur pipette and transferred to a screw cap (foil-lined) vial and stored at −20 C. It should be noted that other solvents may be used instead of *n*-heptane. We have obtained identical results with, for example, *n*-hexane and *n*-octane. However, we do not recommend the use of *n*-hexane because of its toxicity (19).

Extraction of Lipid from Multilamellar Phospholipid Vesicles (MLV)

The efficiency of the SDS extraction method was tested by measuring the lipid extracted from MLV of known composition.

Known amounts of egg lecithin, cholesterol and α-tocopherol were dissolved in dichloromethane. The solvent from a 10 ml sample of this solution was removed under a stream of nitrogen and finally pumped off under vacuum. The MLV were formed by adding 10 ml water to the residue and vortex-stirring. Lipid was extracted by the SDS method from 1 ml aliquots of the MLV using 1 ml of SDS solution, 2 ml of ethanol and 2 ml of *n*-heptane. A reference sample of lipid was prepared by removing the solvent from a 1-ml aliquot of the dichloromethane stock solution and redissolving the residue in 2 ml of *n*-heptane. This reference sample was stored at −20 C in a tightly stoppered vial, and was later used to obtain reference values for the fatty acids, cholesterol and α-tocopherol.

Extraction of Lipid from Plasma

The conventional method for extracting plasma (20) is very similar to the SDS method. The recoveries of lipid obtained by the two methods were compared so that the efficiency of the SDS procedure could be evaluated.

Lipid was extracted in the conventional manner by mixing water (1 ml), ethanol (2 ml) and plasma (1 ml) in a glass tube. *n*-Heptane (1 ml) was then added and the mixture was vortex-stirred for 30-60 sec. The aqueous and organic layers were separated by brief centrifugation.

The SDS method was applied in exactly the same way using the same quantities of material but replacing the water with SDS solutions (1 ml) of different concentrations.

Extraction of Lipid from RBC

RBC, freed of plasma and the buffy coat after washing 3 times in 5 mM phosphate-buffered saline (pH 8.0) in the usual manner (21), were resuspended in the same buffer (hematocrit ca. 50%). Then 5 ml-samples of this suspension were lysed by dropwise addition to ca. 30 ml of 5 mM phosphate buffer (pH 8.0) contained in centrifuge tubes (13,21,22). The hemolysate was spun at 20,000 rpm for 10 min in a Sorvall RC2-B centrifuge equipped with a fixed angle SS-34 rotor (4.25 in. radius). Most of the supernatant was removed from each tube. The red-colored, hemoglobin-contaminated RBC ghost pellet that remained in each tube (ca. 2 ml) was transferred to a separate

16 ml test tube and 2 ml of 5 mM phosphate buffer/5 mM ascorbate (pH 7.0) was added, this being followed by the addition of 1 ml of an SDS solution of known concentration. Under these conditions with this quantity of RBC ghosts, the suspension became transparent when the concentration of the added SDS solution was 0.08 M or greater. Next, 5 ml of ethanol and 2 ml of *n*-heptane were added and mixed with the SDS/RBC ghost mixture in the same way as already described for the general SDS extraction procedure. The *n*-heptane layer that was obtained after centrifugation was colorless.

For comparative purposes, lipid also was extracted from the RBC ghosts by the Folch method that we have used previously (13) and that Nelson has reported extracts lipid very efficiently from plasma and whole RBC (23, 24). However, it is important to use hemoglobin-free ghosts in order to avoid the co-extraction of iron-containing pigments which can cause an almost complete loss of vitamin E (13). A sample of the same (red) ghost suspension used for the SDS extraction experiment was therefore washed twice following the improved procedure developed previously in our laboratory (22). The suspension of (now) white ghosts (ca. 2 ml) was added dropwise to stirred, ice-cold methanol (33 ml) and two portions of cold chloroform (33.5 ml each) were then added. The mixture was magnetically stirred for several minutes and then filtered onto ca. 2 g of Sephadex G-25 (coarse) in a round-bottomed flask. The ghost residue was rinsed with chloroform and the combined filtrate was concentrated by rotary evaporation under reduced pressure. In order to ensure the complete removal of water from the extract by the Sephadex, a further 10-ml portion of chloroform was added to the Sephadex residue and then removed again under reduced pressure. This was repeated twice more. The lipid was finally recovered from the Sephadex by extracting the latter with two 50-ml portions of chloroform and filtering. The combined filtrate was concentrated by evaporation under reduced pressure at a temperature below 30 C and finally to dryness with a stream of nitrogen. The lipid residue was redissolved in 2 ml of *n*-heptane.

The lipid extracts obtained by the new SDS and by the Folch methods were analyzed by TLC on silica gel in order to compare the recovery of the different types of phospholipid. The plates were developed in chloroform/methanol/acetic acid/water (25:15:4:2, v/v/v/v) (25) and the spots were visualized by exposing the plates to iodine vapor.

Extraction of Lipid from Rat Liver

Rat liver homogenate, containing 0.25 g ml^{-1} of liver (wet weight), was extracted by the general SDS method and the Bligh and Dyer method (17,26).

Typically, the SDS procedure used 1 ml of rat liver homogenate, 1 ml of SDS solution, 2 ml of ethanol and 2 ml of *n*-heptane. A partial clarification of the homogenate occurred when the SDS solution was added.

In a typical Bligh and Dyer extraction, 2.5 ml of methanol and 1.25 ml of chloroform were vortex stirred for 2 min with 1 ml of rat liver homogenate. The mixture was centrifuged for 3 min in a clinical centrifuge, and the supernatant was removed with a Pasteur pipette. Then water (1.25 ml) and chloroform (1.25 ml) were added to the supernatant and mixed; this was followed by centrifugation to facilitate separation of the aqueous and organic layers. The aqueous layer was removed by aspiration and the organic layer was then dried over anhydrous sodium sulfate, filtered and evaporated under a stream of nitrogen. The residue was redissolved in 2 ml of *n*-heptane.

Measurement of the Tocopherols

PMHC and 2-BHA were used as non-interfering standards for measuring tocopherol concentrations. Aliquots (50 μl) of *n*-octane solutions of PMHC (8.30 nmol) and 2-BHA (9.23 nmol) were added by micropipette to 500 μl samples of the *n*-heptane lipid extracts. Samples of this solution (100 μl) were injected into a Varian 5000 HPLC instrument equipped with a 250 mm × 4 mm Hibar RT LiChrosorb Si 60 column (Merck) and were eluted with *n*-hexane/*t*-butyl methyl ether (3.0%)/2-propanol (0.05%) at 2 ml min^{-1}. Peaks were detected with a Varian fluorescence detector, equipped with a deuterium lamp, which was connected in series with a Varian Varichrome variable wavelength uv detector set at 295 nm. The fluorescence detector was equipped with a 220 nm interference excitation filter and the emission filter was a 2 mm thick Schott UG-1 glass band filter which gave 89% transmittance at λ_{max} ca. 358 nm and had a 279-419 nm "window" (10% transmittance limits). This emission filter replaced the 5 mm thick Corning 7-60 filter used earlier (13) and gave a more than 3-fold enhancement of the signal. The HPLC and associated detectors were interfaced with a Varian Vista CDS 401 control station and data-handling system.

Measurement of O-acyl-derived Fatty Acids and of Cholesterol

The quantity and composition of the fatty

acids in the O-acyl fraction of the lipid extract and the quantity of cholesterol each were determined by GC analysis after duplicate transesterification of the lipid extract (27).

Fatty Acid Methyl Esters

Transesterification was carried out by heating a mixture of 250-500 µl of the *n*-heptane lipid extract, 1 ml of benzene containing ca. 130 nmol of triheptadecanoin (as internal standard), and 2 ml of anhydrous 0.5 M sodium methoxide in methanol (Supelco) at 80 C for 20 min in a tightly-stoppered glass vial. The mixture was then allowed to cool and 0.1 ml of glacial acetic acid was added, followed by 5 ml of water. After extraction with three 5-ml portions of *n*-hexane, the combined hexane extracts were dried over anhydrous sodium sulfate containing 10% solid potassium bicarbonate. Following filtration, the filtrate was concentrated by evaporation under a stream of nitrogen.

The methyl esters were analyzed on a Varian 3700 GC instrument equipped with a flame-ionization detector using a 1.8 m × 3.2 mm (o.d.) stainless-steel column packed with 10% Silar-9CP on 100-120 mesh Chromosorb W-HP at a helium flow rate of 30 ml min^{-1}. The oven temperature was maintained at 150 C for 5 min and was then increased to 225 C at 3 C min^{-1}. The data were analyzed with a Varian Vista CDS 401 system.

The quantity of each fatty acid methyl ester was measured by comparison with the known quantity of methyl heptadecanoate formed from the triheptadecanoin. It was assumed that each fatty acid methyl ester gave the same peak area per unit weight, an assumption that was fully supported by results obtained with standards containing known amounts of the major fatty acid esters. Total fatty acid values were calculated for the acid form of the ester and included all the minor peaks (e.g., 16:1, 18:3, 20:3, 22:5). The percentage fatty acid composition was, however, based on a calculation restricted to the 16:0, 18:0, 18:1, 18:2, 20:4, and 22:6 set of fatty acids which together constituted well over 80% of the total fatty acid in most samples. Incidentally, for all the O-acyl lipid extracts examined in this work total fatty acid values, which are given in µmol ml^{-1}, can be converted to mg ml^{-1} by dividing by either 3.41 ± 0.03 for plasma, RBC and rat liver or by 3.62 ± 0.03 for MLV.

Cholesterol

Approximately 300 nmol of the internal standard, cholestane, were added to the *n*-hexane extract obtained after transesterification. The cholesterol was measured by GC using a 0.45 m × 3.2 mm (o.d.) stainless-steel column packed with 5% OV 101 on 80-100 mesh Chromosorb W-HP at 230 C with a helium flow rate of 30 ml min^{-1}.

Tests for Presence of SDS in *n*-Heptane Lipid Extracts

These tests were carried out by spotting the lipid extract onto a silica gel TLC plate and developing with dichloromethane/methanol 8:1 (v/v) (28). The TLC plate of an SDS lipid extract from RBC was developed with iodine vapor, and the plate of an SDS lipid extract from rat liver was developed by spraying with 5% sulfuric acid and heating at 80 C on a hot plate.

RESULTS

SDS Extraction of Lipid from MLV, Plasma and RBC Ghosts

The effect of SDS concentration upon the extracted amounts of α- and γ-tocopherol, as measured directly in the *n*-heptane extract, and cholesterol and O-acyl total fatty acid, as measured after the base-catalyzed transesterification, are shown for MLV, plasma and RBC ghosts in Figures 1, 2 and 3, respectively. The

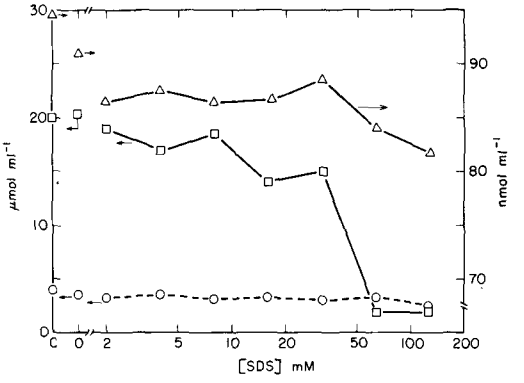

FIG. 1. Effect of final, total SDS concentration (after the addition of ethanol) on the recovery of α-tocopherol (Δ——Δ), total O-acyl fatty acid (□——□) and cholesterol (○ – – ○) from an aqueous MLV suspension by the SDS method (see Materials and Methods). Concentrations of extracted lipids are given per ml of aqueous MLV suspension (the α-tocopherol in nmol ml^{-1}, the total O-acyl fatty acid and cholesterol, which were determined after base catalyzed transesterification, in µmol ml^{-1}). The data points at zero SDS concentration refer to extraction of the MLV suspension without SDS, while those on ordinate C refer to the control analysis of the dichloromethane stock solution used in preparing the MLV. The total O-acyl fatty acid can be converted from µmol ml^{-1} to mg ml^{-1} by dividing by 3.62 ± 0.03. Arrows indicate appropriate ordinate.

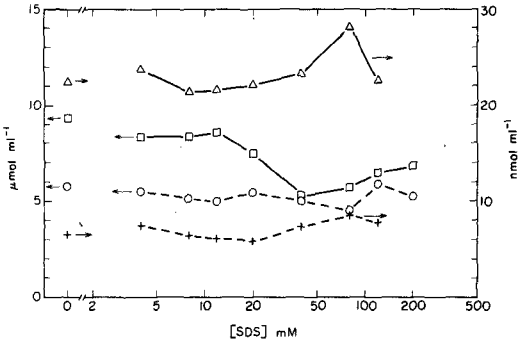

FIG. 2. Effect of final, total SDS concentration (after the addition of ethanol) on the recovery of α-tocopherol (△——△), γ-tocopherol (+---+), total O-acyl fatty acid (□——□) and cholesterol (○——○) from plasma (0.25 ml per ml of aqueous ethanol) by the SDS method (see Materials and Methods). Concentrations of extracted lipids are given per ml plasma (the tocopherols in nmol ml^{-1}, the total O-acyl fatty acid and cholesterol, which were determined after base catalyzed transesterification, in μmol ml^{-1}). The 4 data points corresponding to zero concentration of SDS are reference values obtained by the conventional extraction procedure. The total O-acyl fatty acid can be converted from μmol ml^{-1} to mg ml^{-1} by dividing by 3.41 ± 0.03. Arrows indicate appropriate ordinate.

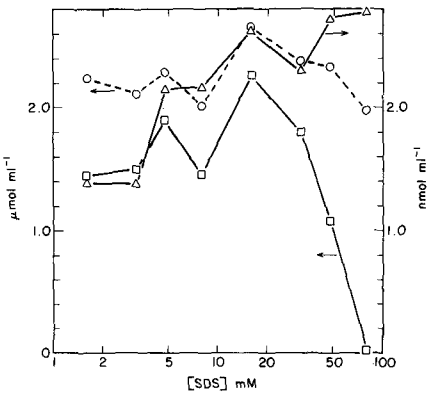

FIG. 3. Effect of final, total SDS concentration (after the addition of ethanol) on the recovery of α-tocopherol (△——△), total O-acyl fatty acid (□——□) and cholesterol (○— —○) from crude (hemoglobin contaminated) RBC ghosts (at a concentration corresponding to 0.25 ml packed RBC per ml of aqueous ethanol) by the SDS method (see Materials and Methods). The ghosts (ca. 2 ml) were derived from 2.5 ml of packed RBC (ex Red Cross) and were extracted with 2 ml of 5 mM phosphate buffer – 5 mM ascorbate (pH 7)/1 ml of SDS solution/5 ml ethanol/ and 2 ml *n*-heptane. Concentrations of extracted lipids are given per ml of packed RBC (the α-tocopherol in nmol ml^{-1}, the total O-acyl fatty acid and cholesterol, which were determined after base catalyzed transesterification, in μmol ml^{-1}). There are no reference points at zero SDS concentration. All adjacent points have been connected, but it seems probable that some of the hills and valleys are experimental artifacts. The total O-acyl fatty acid can be converted from μmol ml^{-1} to mg ml^{-1} by dividing by 3.41 ± 0.03. Arrows indicate appropriate ordinate.

effects of SDS concentration on the corresponding fatty acid compositions are shown in Table 1.

MLV

As can be seen in Figure 1, α-tocopherol and cholesterol are very efficiently extracted by the SDS method. The reference points at C are based on analysis of the dichloromethane stock solution, and those at 0 SDS concentration on the results of an extraction without SDS. At SDS concentrations above 32 mM, the recovery of the fatty acids dropped dramatically, this drop coinciding with a clearing of the suspension after the ethanol was added.

Plasma

The data points in Figure 2 corresponding to zero concentration of SDS are the reference values that were obtained for lipid extracted in the conventional manner (i.e., with ethanol and *n*-heptane only).[2] It is against these points that the values obtained with SDS must be compared. It is apparent that the α- and γ-tocopherol, and cholesterol values are practically independent of the SDS concentration and are in satisfactory agreement with the reference values. (The somewhat greater scatter of the cholesterol values may reflect the fact that cholesterol is measured after chemical manipulation and that the internal standard is added after the transesterification.) The total fatty acid also agrees well with the reference value, provided the final SDS concentration in aqueous ethanol does not exceed ca. 12 mM. However, at higher SDS concentrations there is a fairly abrupt decline in total fatty acid to a rather lower value. This effect was observed consistently for plasma samples obtained from a number of different sources. Examination of Table 1 shows that this change in total O-ocyl fatty acid values is accompanied by significant changes in the composition of the fatty acids.

RBC Ghosts

In Figure 3 are shown the results obtained at various SDS concentrations using RBC obtained from a Red Cross blood sample. Once again, the

[2] We have shown by a comparison of vitamin E, O-acyl fatty acid, cholesterol and phosphorus values that extraction of plasma with ethanol/*n*-octane is at least as efficient as the Folch method. The latter method has been reported to provide near-quantitative yields of lipid (23,24).

TABLE 1

Effect of SDS Concentration on the Fatty Acid Composition of O-acyl Lipid Extracted from MLV, Plasma and RBC Ghosts

	SDS[a] (mM)	O-Acyl fatty acid[b] (μmol ml^{-1})	Percentage composition[b] (wt %) 16:0	18:0	18:1	18:2	20:4	22:6
MLV[c]	Control[d]	20.0	32	11	35	17	5	1
	0[f]	20.3	31	10	38	17	3	1
	2[f]	19.0	30	10	38	17	4	1
	4[f]	17.1	31	11	37	17	3	<1
	8	18.4	30	11	39	17	3	1
	16	13.9	30	11	38	17	4	1
	32[g]	15.1	34	10	37	16	3	<1
	64	1.8[h]	–	–	–	–	–	–
	128	1.9[h]	–	–	–	–	–	–
Plasma[c]	0.0	9.38	19	8	23	43	8	t
	4.0	8.30	18	7	25	41	8	t
	8.0	8.42	19	7	25	41	8	1
	12.0	8.58	19	6	25	42	7	t
	20.0	7.44	19	7	24	42	8	t
	40.0	5.60	17	3	27	48	5	t
	80.0	5.90	16	2	28	48	6	t
	120.0	6.52	16	3	28	48	6	t
	200.0	6.81	16	2	29	47	6	t
		Change[e]:	−3	−6	+6	+4	−2	
RBC ghosts[c]	1.6	1.45	20	22	23	13	19	3
	3.2	1.50	20	22	22	14	19	3
	4.8	1.90	20	19	23	14	20	4
	8.0	1.46	21	19	23	14	20	3
	16.0	2.26	20	19	22	14	20	4
	32.0	1.80	19	20	23	13	21	4
	48.0	1.08	16	19	27	10	23	5
		Change[e]:	−4	−3	+4	−3	+4	+2

[a] Final concentration after addition of ethanol.

[b] Sum of all fatty acids from all O-acyl lipid. See Materials and Methods for details on measurement and calculation (t = trace).

[c] Results correspond to data points in Figure 1 (MLV), Figure 2 (plasma) and Figure 3 (RBC).

[d] Results obtained directly from an aliquot of dichloromethane stock solution (see Fig. 1 legend).

[e] Difference in composition of lipid fatty acid for highest and lowest SDS concentrations.

[f] Layer separation for these samples was difficult. The first fraction of the *n*-heptane extract, obtained in the usual way, was combined with a second fraction obtained after standing overnight at 4 C followed by centrifugation. The difficulty is caused by an emulsion and apparent precipitate at the interface.

[g] A clear solution was obtained after the addition of ethanol at this concentration of SDS and higher.

[h] Percentage composition values are not given because not all components were present in detectable amounts.

cholesterol value appears to be essentially independent of the SDS concentration, though no reference data (i.e., an extraction without SDS) were obtained with this particular blood sample. In contrast, the yield of extracted α-tocopherol is low at low SDS concentrations and does not reach its maximum until the final aqueous ethanolic SDS concentration is ca. 16 mM, thereafter remaining more-or-less steady. The total fatty acid extracted also increases slightly to ca. 16 mM SDS, but then declines rapidly to zero at higher concentrations of SDS. It can be seen from Table 1 that the rapid decline in the total fatty acid value is accompanied again by an appreciable change in its composition.

The SDS and Folch extraction procedures are compared in Table 2 for RBC ghosts prepared from a sample of fresh blood. The SDS method is clearly superior for the extraction of α-tocopherol. (Note that if the RBC ghosts are contaminated with hemoglobin, i.e., are red in color, then phosphate-buffered ascorbate should be added prior to the SDS to avoid a potentially substantial loss of α-tocopherol.) The cholesterol values, which again vary little with SDS concentration, show that recoveries by the SDS method are at least as good as by

TABLE 2

Comparison of Lipid Extracted from RBC Ghosts by the Folch and SDS Methods[a]

Method	α-Tocopherol[b,c] (nmol ml^{-1})	Cholesterol[b,d] (μmol ml^{-1})	O-Acyl lipid: Total fatty acid[b,d] (μmol ml^{-1})	Percentage composition[d] (wt %) 16:0	18:0	18:1	18:2	20:4	22:6
Folch	1.84 ± 0.09	2.67 ± 0.13	2.57 ± 0.14	22	19	22	13	19	4
SDS (mM)[e]: 13	3.09 ± 0.43	3.06 ± 0.13	2.80 ± 0.03	22	20	22	13	20	3
27	3.15 ± 0.50	3.29 ± 0.10	2.80 ± 0.03	21	21	21	11	21	6
40	3.04 ± 0.67	2.62 ± 0.08	2.46 ± 0.03	20	21	21	12	21	5
80	2.56 ± 0.51	3.03 ± 0.10	0.44 ± 0.03	16	25	21	8	25	4

[a]Fresh blood sample from AW. SDS extractions were carried out on crude ghosts (without added phosphate-buffered ascorbate), and the Folch extraction was carried out on washed ghosts, as described in Materials and Methods.

[b]Lipid values refer to concentrations in 1 ml of packed RBC.

[c]Determined by HPLC. Each value is the mean and difference from the mean of the 2 results obtained consecutively with the fluorescence and UV detectors, respectively.

[d]Determined by GC after base-catalyzed transesterification (see Materials and Methods). Each cholesterol and total fatty acid value is the mean and difference from the mean of 2 results.

[e]Final concentration of SDS after the addition of ethanol. The concentration of ghosts corresponds to 0.25 ml of packed RBC per ml of aqueous ethanol, i.e., a 4-fold dilution of the original packed RBC.

the Folch method and perhaps slightly better. With final SDS concentrations in the 13-40 mM range, the total fatty acid extracted and its composition agree well with the results obtained by the Folch method. The similarity in composition suggested that the same types of phospholipid were being extracted in the same proportion by both the SDS and Folch methods. This was confirmed by TLC for PC, PE, PS and SP, which were found to be present in qualitatively similar proportions in lipid extracted by the two methods. At high SDS concentrations there is, once again, a drastic decrease in the total fatty acid and a pronounced change in its composition.

SDS was not found by TLC in the *n*-heptane extract of lipids from RBC ghosts.

SDS Extraction of Lipid from Rat Liver Homogenate

The results presented in Table 3 show that rat liver homogenate can be extracted successfully by the SDS method, and that this method is clearly superior not only in time and labor but also in terms of its recovery of α-tocopherol, cholesterol and probably total fatty acid, compared to the Bligh and Dyer method. Note that recoveries are insensitive to a remarkably wide range of SDS concentrations (ca. 12.5-ca. 50 mM in the aqueous ethanol). With this type of tissue, as with the others we have examined, too high a concentration of SDS (>50 mM) adversely affects the total fatty acid recovered and, once again, causes a dramatic change in the fatty acid composition (Table 3).

SDS was not found by TLC in an *n*-heptane lipid extract.

Other Observations Pertinent to the SDS Extraction Method

Lipid extracted from RBC ghosts and rat liver by the SDS method generally gave significantly "cleaner" HPLC traces when analyzed for vitamin E than did the corresponding Folch or Bligh and Dyer lipid extracts. (Also, the problem with over-long induction periods that we have occasionally experienced in the inhibited-autoxidation method (13) has not been encountered with lipid extracted from hemoglobin-free ghosts, though it may occur in lipid extracted from hemoglobin-contaminated, ascorbate-protected ghosts (13).) Extractions performed with SDS obtained from Bio-Rad showed a fluorescent impurity in the HPLC chromatograms which did not, however, interfere with the α-tocopherol or internal standard peaks. This impurity was not present in the SDS from BDH.

The effects of omitting SDS or ethanol or both upon lipid recovered from MLV are presented in Table 4. It can be seen that a substantial proportion of the lipid can be extracted with *n*-heptane alone, i.e., without the addition of SDS or ethanol, although in this case it is more difficult to obtain a separation of the aqueous and organic layers. (This problem was even more severe when an attempt was made to extract plasma lipid with *n*-heptane alone.) If the SDS extraction procedure is used

TABLE 3

Effect of SDS Concentration upon Recovery of Lipid from Rat Liver Homogenate Comparison with Bligh and Dyer Extraction[a]

Method	α-Tocopherol (nmol g^{-1})	Cholesterol (μmol g^{-1})	O-Acyl lipid: Total fatty acid (μmol g^{-1})	Composition (wt %) 16:0	18:0	18:1	18:2	20:4	22:6
Bligh-Dyer (1)	27 ± 12	4.56 ± 0.10	62.4 ± 0.7	20	18	10	25	21	7
Bligh-Dyer (2)	20 ± 6	3.52 ± 0.10	52.2 ± 5.1	19	19	8	25	24	5
SDS (mM)[b]: 12.5	36 ± 9	5.49 ± 0.10	57.3 ± 3.4	19	20	8	22	26	5
20.0	35 ± 10	6.00 ± 0.31	67.9 ± 4.1	18	20	8	23	26	6
25.0	34 ± 8	5.80 ± 0.21	65.1 ± 4.1	18	19	8	22	25	7
50.0	35 ± 9	5.90 ± 0.31	59.3 ± 1.7	18	20	8	24	26	4
100.0	37 ± 11	5.49 ± 0.10	24.9 ± 0.7	17	16	11	30	21	5
150.0[c]	40 ± 15	4.45 ± 0.16	9.9 ± 0.3	24	3	21	34	11	6
			Change	+5	−17	+13	+12	−15	+1

[a]Lipid concentrations refer to 1 gm of liver (wet weight). The aqueous homogenate contained 0.25 gm liver per ml. α-Tocopherol, cholesterol and O-acyl lipid were determined in the usual way (see Materials and Methods).

[b]Final SDS concentration after the addition of ethanol. The homogenate (1 ml) was extracted with SDS solution (1 ml; 0.05-0.40 M)/ethanol (2 ml)/*n*-heptane (2 ml) (see Materials and Methods). The final liver concentration was 63 mg ml^{-1}.

[c]1 ml of homogenate was extracted with 0.4 M SDS (3 ml)/ethanol (4 ml)/heptane (2 ml). The final liver concentration was 31 mg ml^{-1}.

TABLE 4

The Effect of Omitting Ethanol or SDS, or Both, upon Recovery of Lipid from MLV[a]

	SDS: Volume (ml)	SDS: Final concentration (mM)	Ethanol (ml)	Water (ml)	α-Tocopherol (nmol ml^{-1})	Cholesterol (μmol ml^{-1})	O-Acyl fatty acid (μmol ml^{-1})
Control[b]	–	–	–	–	94.0	3.79	20.0
MLV[c]	1	2	2	0	86.6	3.18	19.0
MLV[c]	0	0	2	1	91.0	3.47	20.3
MLV[d]	0	0	0	0	79.2	3.68	16.8
MLV[e]	1	40	0	0	–[e]	–[e]	–[e]

[a]Volumes of SDS solution, ethanol and water indicate the amounts of each that were added to 1 ml of aqueous MLV suspension containing α-tocopherol, cholesterol and egg lecithin. Lipid was extracted into 2 ml of *n*-heptane (see Materials and Methods). Lipid concentrations refer to 1 ml of aqueous MLV suspension.

[b]Lipid values were determined directly from a 1 ml aliquot of the dichloromethane stock solution (see Materials and Methods).

[c]Layer separation was not readily obtained. See footnote "f," Table 1.

[d]Required 15 min centrifugation.

[e]Lipid values could not be determined because the emulsion did not separate into distinct aqueous and organic layers during centrifugation.

on the MLV, then ethanol cannot be omitted since, without ethanol, the lipid/SDS/*n*-heptane emulsion does not separate clearly into two phases on centrifugation (Table 4).

Although plasma lipids can be successfully extracted without SDS using only ethanol and *n*-heptane, our attempts to extract crude RBC ghosts in the same way were unsuccessful.

For quantitative work one must know the volume of the organic phase containing the extracted lipid. In systems employing equal volumes (2 ml) of aqueous solution, ethanol and *n*-heptane, the recovery of the organic layer was found to be 95-97.5% (1.90-1.95 ml).

DISCUSSION

General Considerations: SDS Method vs. Folch or Bligh and Dyer Methods

The control experiments conducted with plasma and MLV, as well as the comparisons with the extraction results obtained by the

traditional methods of Folch for RBC ghosts and Bligh and Dyer for liver homogenate, show very clearly that the SDS method as outlined herein provides an extremely rapid (≤5 min), mild, clean and efficient method for extracting lipid.

Within certain upper and lower limits, the values for which depend upon both the type of lipid and the nature of the lipid extracted, the recovery of lipid is independent of the SDS concentration.

These limits are widest for rat liver (0.25 g of liver suspended in 4 ml of aqueous ethanol, see Table 3). For α-tocopherol no upper or lower limits were established, and the yield of cholesterol decreased slightly at final SDS concentrations greater than 100 mM, while the yield of O-acyl lipid decreased fairly precipitously at SDS concentrations greater than ca. 50 mM. Although the recovery of O-acyl lipids from RBC imposes narrower limits on the concentration of SDS, these limits are nevertheless sufficiently broad to be useful for routine extraction of total lipid.

As a practical guide, RBC may be extracted by successively adding and mixing SDS (1 ml; 40-80 mM), ethanol (2 ml) and *n*-heptane (1-2 ml; vortex stir for 30-60 sec) with crude ghosts suspended in 5 mM phosphate/5 mM ascorbate (ca. 1 ml; pH 7; packed RBC volume originally 1 ml), followed by brief centrifugation. Rat liver homogenate (1 ml; 1 part liver to 3 parts water) is extracted in the same way using SDS (1 ml; 40-200 mM), ethanol (2 ml) and *n*-heptane (1 ml).

The "cleanness" of the SDS method is apparent not only in the significant reduction of contaminants in the HPLC analyses for vitamin E and by the absence of TLC-detectable amounts of SDS in the *n*-heptane extracts, but also from the fact that the *n*-heptane extracts of RBC ghosts heavily contaminated with hemoglobin are colorless, i.e., the iron-containing porphyrins that normally contaminate the RBC lipids extracted with chloroform/methanol are excluded from the SDS/ethanol/*n*-heptane lipid extract.

Preliminary experiments indicate that vitamin E can be extracted directly from either fresh or frozen RBC by the SDS method without first making ghosts. For example, rat RBC (1.0 ml; hematocrit ca. 50%) suspended in 5 mM ascorbate/5 mM phosphate-buffered saline (1 ml) were treated with SDS (5 ml; 0.1 M), ethanol (7 ml), and *n*-heptane (1-2 ml). The recovery of vitamin E was found to be the same for both fresh RBC and RBC stored frozen in the ascorbate/phosphate-buffered saline.

The amount of *n*-heptane used is not critical. This offers the potential for a modest, initial concentration of lipids by using a volume of *n*-heptane which is less than the volume of the original tissue. Other alkanes can be substituted for *n*-heptane, and it seems likely that certain other combinations of solvents also could be employed successfully. However, the dependence of lipid recovery upon the proportion of ethanol used and the effect of ethanol substitution by other alcohols have not been investigated.

The successful application of the SDS method to rat liver homogenate strongly suggests that the method has general applicability. A preliminary confirmation of the generality, speed and efficiency of the method has been obtained using aqueous homogenates of lung, heart, muscle, kidney, testes and brain tissue from a rat. The extractions of all six of these homogenates were accomplished in 20 min (1 ml homogenate/1 ml 0.1 M SDS/2 ml ethanol/2 ml *n*-heptane), and the results for α-tocopherol, cholesterol and O-acyl fatty acid were all at least equal to the results obtained by the method of Bligh and Dyer. Details of this work will be published at a later date.

It seems likely that the SDS method also could be used for the extraction and analysis of other minor, but important, lipid components such as β-carotene, ubiquinone and vitamins A, D and K.

We also have explored the use of Triton X-100 but found it to be much less satisfactory than SDS both because of poor phase separation and because there were a multitude of unidentified peaks in the HPLC chromatogram.

Factors Affecting Recovery and Composition of Lipid

Studies of the solubilization of pure PC vesicles and of biological membranes with detergents have provided evidence for differential complexation of the various components of membranes and for the eventual formation of soluble mixed lipid-detergent micelles and lipid-protein-detergent complexes (29-36). These observations help explain some of our experimental results. For example, the recovery of total O-acyl lipid from MLV and RBC declined to zero at the higher concentrations of SDS (Figs. 1 and 3), whereas its recovery from plasma declined by only ca. 30-40% and then reached an approximately constant value (Fig. 2). We suggest that these results are due to the fact that when there is sufficient SDS present to produce mixed lipid-SDS micelles, the more polar phospholipid is preferentially retained in these micelles upon addition of ethanol and *n*-heptane, whereas the less polar lipids (e.g.,

tocopherol, cholesterol, cholesteryl esters and triglycerides) are partitioned predominantly into the *n*-heptane. Since plasma contains triglycerides and cholesteryl fatty acid esters but RBC and MLV do not, the total O-acyl fatty acid value does not decline to zero for plasma but does for the RBC and MLV. Presumably the change in composition of the plasma-derived fatty acids with changing SDS concentration (Table 1) reflects differences in the fatty acid composition of the phospholipids, triglycerides and cholesteryl esters.

The dependence of phospholipid recovery upon SDS concentration shows a considerable variation with the nature of the material being extracted. MLV were found to be the most sensitive and the rat liver homogenate the least sensitive. This phenomenon appears to be related to the relative amount of protein present in each material. This is not too surprising since it is known that SDS binds strongly to proteins (29,30,37-40). We suggest that the protein acts as a kind of "buffer" or "sponge" which allows a considerable quantity of SDS to be added before the concentration of free, monomeric SDS reaches the CMC.[3] This will occur only after complete saturation of the protein. Rat liver shows the greatest range of acceptable SDS concentrations because it contains the most protein, while the "buffer" effect is inoperative in MLV because they contain no protein.

It will be clear from the foregoing that the upper SDS concentration limit at which phospholipid recovery begins to decline will depend to some extent on the tissue extracted, since it depends on the concentrations of both lipid and protein.

The marked changes that occur in the total fatty acid composition at the higher SDS concentrations that are associated with declining phospholipid recovery from plasma, RBC and rat liver (Tables 1, 2 and 3) show that the nature of the phospholipid affects its partitioning between the mixed micelles and the *n*-heptane. This effect can be attributed, in part, to differences in the nature of the phospholipid head groups, since it has been shown (36) that there are differences in the rates of SDS solubilization of PC, PE, PS and SP from RBC ghost membranes. Results obtained with MLV at concentrations of SDS associated with rapidly declining O-acyl lipid recovery (data not shown) indicate that the nature of the fatty acid tail must also play some role since the MLV contain only one type of phospholipid, PC. These latter results indicate that retention in the mixed micelles of the aqueous phase is favored for PC containing palmitic acid and is disfavored for PC containing arachidonic acid.

CONCLUSION

The results presented here indicate that the combination of the well-known property of detergents to dissociate and solubilize membrane proteins (29,30,37,38,44,45) with the extraction capabilities of aqueous alcohol/alkane mixtures[4] provides a promising alternative for general lipid extraction. The mildness and speed of the new method are important for the recovery of small quantities of labile compounds such as vitamin E. Provided the concentration of SDS is kept within certain rather broad limits, excellent recoveries of O-acyl lipid are obtained.[5] Higher concentrations of SDS appear to offer a way of separating phospholipids from less polar compounds such as cholesterol, cholesteryl esters, triglycerides, tocopherols, etc., as indicated by the results obtained for plasma and MLV.

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[3] Even in the absence of protein and lipid, the CMC will not be the same as in water since it is influenced by ethanol (41,42) and by ions from buffers and dissolved salts (29,42,43).

[4] We have shown by a comparison of vitamin E, O-acyl fatty acid, cholesterol and phosphorus values that extraction of plasma with ethanol/*n*-octane is at least as efficient as the Folch method. The latter method has been reported to provide near-quantitative yields of lipid (23,24).

We have found that ethanol/hexane quantitatively extracts α-tocopherol dissolved in aqueous SDS micelles.

Alcohol/alkane mixtures have been used previously for the quantitative recovery of neutral lipid from liver (46) and microsomes (47) and also have been found to be suitable for replacement of chloroform/methanol in the Folch method (48).

[5] Although a rapid procedure for specifically measuring vitamin E in tissue and plasma using acetone has been reported recently (49), the insolubility of phospholipids in acetone precludes the use of this method for determining levels of peroxidizable lipid.

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Rapid and Convenient Separation of Phospholipids and Non Phosphorus Lipids from Rat Heart Using Silica Cartridges

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ABSTRACT

The separation of non phosphorus lipids and phospholipids of rat heart using Sep-pack Silica cartridges is described. No cartridge preparation is necessary before utilization. The separation of lipid extracts is very fast. A complete partition of non phosphorus lipids and phospholipids is obtained.
Lipids 20:40-41, 1985.

INTRODUCTION

Borgström (1) showed that the non phosphorus lipids (NPL) and phospholipids (PL) could be separated by adsorption chromatography on a silica column. Using this method, the non phosphorus lipids are eluted by chloroform and the phospholipids by methanol. A number of modifications (2,3) were introduced to this method. The method described by Comte (4) permits 30mg of lipid extract to be separated on a glass column containing 1g of silica and 0.5g of celite 545; the fractions were eluted with 20ml of chloroform followed by 20ml of methanol.

This technique is time consuming: 2 to 3 hr of column preparation and 2 to 3 hr of separation. The use of the silica cartridges, 25 mm x 10 mm I.D., (Sep-pack, Waters S.A., Framingham, Massachusetts) eliminates the first step and reduces the second process to 5 min.

EXPERIMENTAL PROCEDURES

- NPL and PL Separation

This separation was tested on the total heart lipids of SPF male Wistar rats (16 weeks of age, mean body weight 380 g) fed a commercial diet (UAR 113).

The lipids were extracted by the method described by Folch et al. (5). The extract was evaporated to dryness at 40 C in a rotary vacuum apparatus, left overnight in a vacuum dessicator and then weighed. It was then diluted in chloroform to obtain a solution containing about 30mg of lipids in 500 µl of solvent.

A sample was injected at the top of the cartridge, using a 500µl syringe. After adsorption of the sample, a syringe containing 20ml of chloroform was connected to the top of the cartridge. It was very important to avoid the formation of air bubbles between the top of the cartridge and the solvent. Chloroform was pushed through the cartridge (about 25 ml/min) and the fraction containing the NPL collected. Similarly, the fraction containing the PL was eluted with 30ml of methanol.

The fractions were dried as indicated above, then weighed, in order to determine the respective quantities of NPL and PL.

Purity of the Fraction

The purity of the 2 fractions was verified

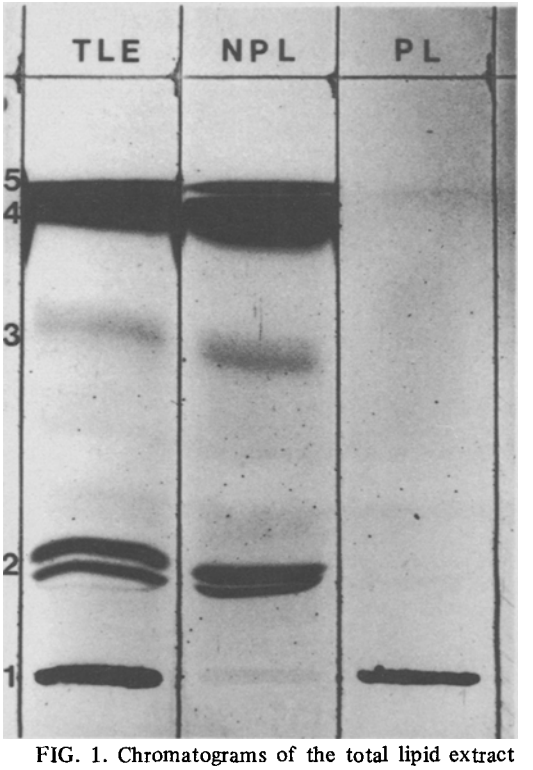

FIG. 1. Chromatograms of the total lipid extract (TLE) and the 2 fractions (PL and NPL). 1 = phospholipids; 2 = diglycerides and cholesterol; 3 = free fatty acids; 4 = triglycerides, and 5 = cholesteryl esters.

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TABLE 1

Comparison of the 2 Separation Methods of NPL and PL of Lipids Extracted from Rat Heart

		Lipid extract	NPL	PL
Separation according to J. Comte (4)	a	27.5	6.2 ± 0.04	20.7 ± 0.05
	b	534.36	N.D.*	532.78 ± 2.012
Separation using Sep-pack cartridges	a	27.5	6.4 ± 0.05	20.6 ± 0.06
	b	534.36	N.D.*	531.20 ± 2.026

a = mg of lipids (mean ± SEM, n=5).
b = μg of phosphorus (mean ± SEM, n=5).
*N.D. = Not detected.

by thin layer chromatography (TLC) on silica gel plates (Merck A.G., Darmstad, W. Germany). The developing solvent system (6) was a mixture of hexane/diethyl ether/methanol/acetic acid (90:20:5:2, v/v/v/v). The bands were detected by iodine vapor. Also, possible cross contamination of phospholipids in the NPL was tested by a phosphorus assay (7).

RESULTS AND DISCUSSION

Figure 1 shows the fraction eluted by chloroform contained only NPL, whereas the fraction eluted by methanol contained only PL. Moreover, the phosphorus assay did not show any phospholipid contamination in the NPL (Table 1).

The retrials and comparisons of these methods were tested on a pool of heart lipid extracts from rat. The results of these tests are indicated in Table 1. There is no noticeable difference between the 2 methods. Duplication is excellent. No loss of lipid seems to occur after column fractionation; particularly the phospholipid recovery is satisfactory, as shown in Table 1. The only slight variation observed in either probably is due to the inherent errors in this type of separation.

Volumes of 20 and 30ml are suitable for sample sizes ranging from 10 to 100mg. It is necessary to establish the approximate solvent volume, as well as the method of analysis of the resulting fractions, for samples smaller than 10mg. For samples greater than 100mg there probably is a risk of cartridge saturation. We did not test the cartridge in this condition. The solvent flow used in the fractionation does not modify the quality of the separation.

The presence of monoglycerides (MG) in the sample resulted in an impure PL fraction. In this case, it is necessary to use an in-between solvent system consisting of 5ml of a mixture of chloroform/methanol (49:1, v/v) which elutes all MG from the sample.

The cartridges can be reused 5 to 6 times. For that purpose, it is necessary only to wash the cartridge between 2 fractionations with 20ml of methanol, then 20ml of chloroform.

Similar results were obtained with lipids extracted from other tissues such as liver or kidney.

In conclusion, the replacement of traditional glass columns by ready made cartridges gives a noticeable improvement both in ease and in speed for the separation of non phosphorus lipids and phospholipids, without affecting the quality of the final result.

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A Colorimetric Assay of Pancreatic Lipase: Rapid Detection of Lipase and Colipase Separated by Gel Filtration

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ABSTRACT

A rapid assay for pancreatic lipase (E.C., glycerol-ester hydrolase 3.1.1.3) is described. The assay is based on the color change of a pH indicator as butyric acid is released from the substrate tributyrin. A mixture made with tributyrin and the water soluble components of the assay is ideally suited for use as a rapid test as, for example, when assaying chromatography fractions. Quantitative data can be obtained by measuring the disappearance of absorbance at 557 nm versus a blank reaction. The assay has been used in the rapid preparation of colipase-free lipase and colipase.
Lipids 20:42-45, 1985.

INTRODUCTION

Pancreatic lipase has been the object of extensive biochemical and physiological study. For a complete recent review of lipase studies the reader is referred to Verger (1).

Lipase hydrolyzes triglycerides. However, bile salts are able to inhibit this hydrolysis (2-4). Lipase activity can be restored by the action of colipase, a low molecular weight protein cofactor tightly associated with lipase (3).

Because lipase is relatively unstable, time lost while assaying for active fractions during the course of purification can result in substantially lower yields. An assay is described here which can significantly reduce the amount of time involved in locating and quantifying chromatographic fractions with lipase activity. The assay was used to monitor the elution of lipase and colipase from a Sephadex G-100 column used to simultaneously prepare colipase-free lipase and colipase from crude pancreas acetone powder.

MATERIALS AND METHODS

Pancreas acetone powder (PAP), Sephadex G-100, phenylmethylsulfonyl fluoride (PMSF) and sodium taurodeoxycholate (NaTDC) were purchased from Sigma Chemicals (St. Louis, Missouri). Tributyrin was purchased from Eastman Organic Chemicals (Rochester, New York). Phenol red was a product of Matheson, Coleman and Bell (Cincinnati, Ohio) and was prepared as a stock solution of 0.04 per cent as described elsewhere (5).

Assay Mixture

A mixture was prepared which contained 100 μl tributyrin and 1.5 ml phenol red stock solution in 100 ml of water adjusted to pH 9.2-9.3 with NaOH. Because tributyrin is only sparingly soluble in water, the mixture was vigorously stirred on a magnetic stirrer to fully disperse the substrate. To test for the presence of colipase the same assay mixture was prepared containing 6 mM of NaTDC and used with added colipase-free lipase to provide lipolytic activity.

Qualitative Test

Column fractions were scanned routinely for lipase activity by mixing appropriate aliquots (5-10 μl) of each fraction with 0.5 ml of assay mixture and incubating at 37 C for 5 to 10 min. A color change from red to yellow in this time period is taken as an indication of lipase activity.

Colorimetric Assay

Those fractions containing lipase activity as demonstrated with the qualitative test were reassayed by mixing 10-30 μl of the active fractions with 3 ml of assay mixture in 13 x 100 mm disposable test tubes and incubating at 24 C for a prescribed time period (usually 1-5 min). This allows the color change to be quantified by measuring the absorbance at 557 nm using a Bausch and Lomb Spectronic 21 spectrophotometer. With prolonged incubation each assay mixture (even those without enzyme) will turn yellow, due to the spontaneous hydrolysis of tributyrin. For this reason a control tube lacking enzyme is always included in the incubations. Because the change from red to yellow is a result of the decrease in absorbance at 557 nm, spectrophotometric measurements were recorded as (A_{557} control/A_{557} test) so that the resulting plots would give peaks instead of valleys.

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Those fractions having lipase activity were reassayed in the presence of 6 mM NaTDC. Fractions which were lipase negative under these conditions were eonsidered to be free of colipase.

Colipase Assay

To test for colipase activity, an aliquot from one of the lipase fractions previously identified as colipase-free was added to individual tubes of assay mixture containing 6 mM NaTDC. This mixture was then used to assay fractions which were thought to contain colipase. Fractions which contained colipase were able to restore the activity of the bile salt-inhibited lipase.

Autotitration Assay

The assay procedure of Patton et al. (8) was used on a Radiometer (Copenhagen) pH stat. Assay mixtures were 10 ml and the 2.5 ml burette was used to deliver 0.01 M NaOH. One unit of lipase or colipase activity is defined as that amount of enzyme (coenzyme) which causes the release of one μmole of butyric acid per min at pH 7.0 and 24 C.

Chromatography

The procedure used for the preparation of colipase-free lipase (and simultaneously colipase) was a modification of that of Verger et al. (9). All operations were performed at 0-4 C. Fifteen grams of PAP were extracted in 300 ml of 0.1 M Tris-HCl pH 9.0 containing 0.02% sodium azide and 1 mM PMSF (Buffer A) for 1 hr with vigorous stirring. The extract was centrifuged for 15 min at 15,000 × g. The supernatant fluid was brought to 80% saturation with solid ammonium sulfate (48.8 gm/100 ml) and centrifuged at 15,000 × g for 15 min. The resulting pellet was resuspended in 150 ml 5 mM Tris-HCl pH 8.0 containing 3.3 mM $CaCl_2$, 1 mM PMSF and 4 mM NaTDC (Buffer B). The suspension was then brought to 30% (v/v) with n-butanol and 15% (w/v) with ammonium sulfate. After stirring for 1 hr, the suspension was centrifuged at 11,500 × g for 40 min. The creamy interface was collected carefully, suspended in 40 ml Buffer B, then dialyzed versus 2 × 900 ml of the same buffer using 3500 MW cutoff dialyzer tubing from Spectrapor. After about 18 hr of dialysis, the extract was centrifuged for 60 min

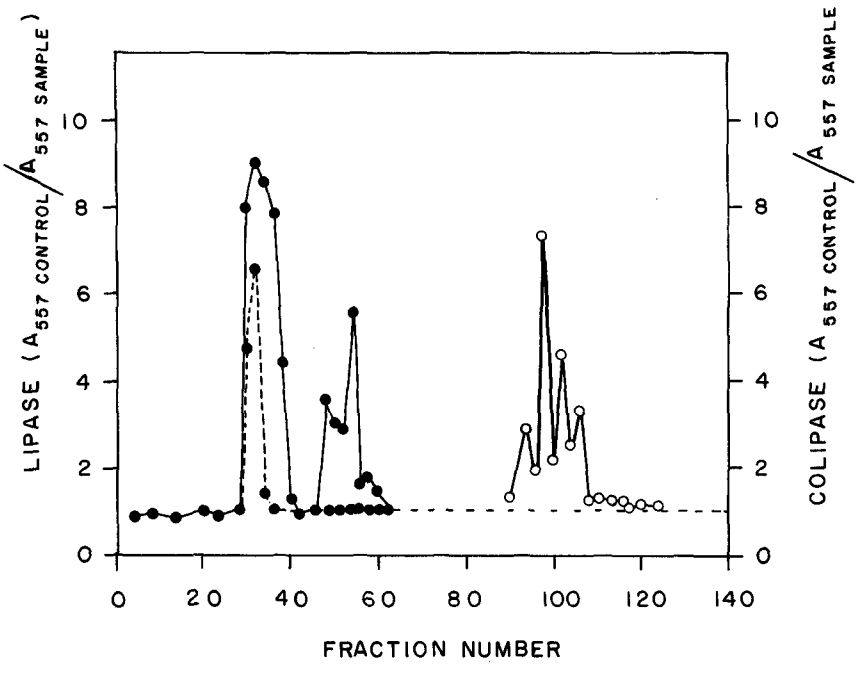

FIG. 1. Sephadex G-100 chromatography, colorimetric assay. PAP prepared as described in Materials and Methods was chromatographed on a 2.5 × 100 cm column of Sephadex G-100 in Buffer C at a flow rate of 0.5 ml/min. Twenty ml of sample was applied to the column and 5 ml fractions were collected. The colorimetric assay was used to monitor lipase and colipase activity, which is expressed as A_{557} control/A_{557} sample. (●—●) = lipase activity (assayed without NaTDC); (● - - - ●) = colipase-free lipase activity (assayed with NaTDC; (○ —○) = colipase activity.

at 11,500 x g. One-third of the supernatant fluid was concentrated by ultrafiltration (Amicon PM 10) and the remaining two-thirds saved for further use. The concentrated lipase preparation was brought to 8 mM NaTDC and applied to a 2.5 x 100 cm column of Sephadex G-100 equilibrated with 10 mM Tris-maleate pH 9.0 containing 150 mM NaCl, 1 mM $CaCl_2$, 1 mM PMSF and 0.02% sodium azide (Buffer C). Five ml fractions were collected.

RESULTS

The delipidated extract of pancreas acetone powder was chromatographed on Sephadex G-100 as described above. The fractions were initially screened for lipase activity by the qualitative test described in Materials and Methods. Fractions indicating lipase activity were reassayed using the colorimetric assay and quantified spectrophotometrically. The results are shown in Figure 1. Two peaks of lipase activity can be seen in the front portion of the chromatogram. When reassayed with 6 mM NaTDC, the earlier eluting of these 2 peaks retains a large portion of activity in the presence of the bile salt, whereas the second peak was completely inhibited, suggesting it was devoid of colipase.

To test for colipase, 10 µl aliquots of lipase from fraction 52 were added to tubes of assay mixture containing 6 mM NaTDC, then 50 µl aliquots of fractions 70-180 were added and the mixtures incubated as described in Materials and Methods. Those fractions which restored the bile salt-inhibited lipase activity are shown in Figure 1. Colipase is well separated from free lipase and from the earlier eluting lipase-colipase complex.

The results of the conventional titrametric assay of a similarly chromatographed crude lipase preparation are shown in Figure 2. The results seen in Figures 1 and 2 are strikingly similar. Non-inhibitable, or colipase bound lipase, elutes in the area around the void volume for this particular column. Bile salt-inhibitable lipase elutes later, indicating smaller molecular weight and colipase elutes well back near the included volume of the column, indicating a small molecular weight. These size classes are consistent with the earlier reports of Morgan et al. (10) and Rathelot et al. (2).

DISCUSSION

A colorimetric assay is described which can be used as a rapid qualitative or semi-quantitative measurement of lipase activity. The assay was used to identify lipase, colipase and the lipase-colipase complex prepared in a single chromatographic step. The lipase/colipase activity profiles obtained after assay of gel filtration fractions of crude pancreas acetone powder are qualitatively the same whether assayed by the standard pH-stat method or by the colorimetric assay. The main difference is one of time—the

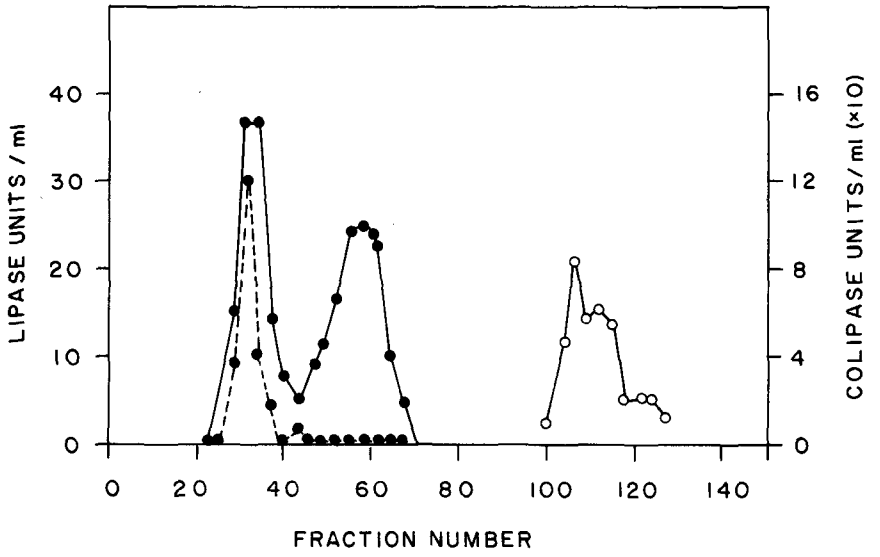

FIG. 2. Sephadex G-100 chromatography, pH-stat assay. All conditions were the same as in Figure 1, except that the column fractions were assayed with the pH-stat assay of Patton et al. (8). (• — •) = lipase activity (assayed without NaTDC); (• - - - •) = colipase-free lipase activity (assayed with NaTDC); (o — o) = colipase activity.

total analysis time for the experiment described in Figure 1 was 2 hr, whereas the same analysis conducted using the titrametric assay (Fig. 2) required about 8 hr. Although only the titrametric assay gives quantitative measurements, for the purpose of identifying activity peaks from a column, the colorimetric assay is entirely suitable. After pooling the desired fractions a single titrametric assay can be performed to obtain precise activity levels for calculation of yield and specific activity. Identification of active fractions is greatly facilitated by using the colorimetric assay as a qualitative test, further speeding up the overall procedure.

The assay mixture used in these experiments is unbuffered (except for the pH indicator) so that the decrease in pH can be followed. One result of using an unbuffered assay mixture is that the reaction rate changes during the course of the assay. Fortunately, for this application, the activity profile of pancreatic lipase has a broad maximum in the range of pH 6.5-8.5 (7) so the change in reaction rate is actually rather small. Thus, despite the fact that the reaction changes rate as the pH drops from above 9 to below 6 during the course of the assay, there remains a roughly linear change (data not shown) in color as butyric acid is released from the substrate. Because of the changing pH and consequent rate change the assay is not satisfactory for absolute quantification of lipase activity, but it is ideal for making relative measurements such as those in this study.

The partial purification described here does not dissociate all of the lipase-colipase found in PAP. However, it is possible to obtain reasonable quantities of free lipase and free colipase in 3 days with a single chromatography step. Although not pure, the lipase and colipase prepared in this manner should be suitable for many types of studies including: kinetic, inhibitor, drug effect, in vitro lipolysis, etc. It also may be possible to covalently attach colipase purified in this manner to an inert support to use in the affinity purification of lipase as described by Patton and Andersson (11).

ACKNOWLEDGMENT

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COMMUNICATIONS

A Diurnal Variation of Hepatic Acid Cholesteryl Ester Hydrolase Activity in the Rat

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ABSTRACT

The diurnal variation in lysosomal acid cholesteryl ester hydrolase (Acid CEH), (EC 3.1.1.13) has been examined in fed, fasted and adrenalectomized rats. The Acid CEH activity of normal rat liver exhibits a diurnal rhythm with maxima at 06.00 hours and minima at 18.00 hours, but such a rhythm was not observed in spleen and brain. This rhythm was abolished after fasting for two days, and the resulting Acid CEH activity remained constant at the minimum level. However, adrenalectomy did not abolish the diurnal rhythm. These results indicate that the Acid CEH activity varies according to a diurnal rhythm with maxima and minima separated by approximately 12 hr. Further, it is evident that the appearance of this rhythm is dependent upon dietary, but not adrenal hormone influence.
Lipids 20:46-48, 1985.

INTRODUCTION

The liver is a major site of cholesteryl ester hydrolysis in the rat, and it is well known that hepatic lysosomal Acid CEH is required for hydrolysis of endogenous and exogenous cholesteryl esters introduced into cells as lipoproteins (1).

This enzyme activity can be markedly reduced by cholesterol feeding (Tanaka, M., unpublished data) and by triton WR-1339 treatment (2), and it seems to be related to the pathogenesis of diseases such as atherosclerosis (3).

Recently we found that a cytosolic protein in rat liver has an inhibitory effect on lysosomal Acid CEH activity (4). In addition, Umezawa et al. (5) have isolated an inhibitor of lysosomal acid lipase from Actinomycetes. Little is known, however, about the regulation of the Acid CEH activity in the liver under various conditions. On the other hand, many mammalian circadian rhythms have been recognized and described in detail; for example, that of 3-hydroxy-3-methylglutaryl-CoA reductase (6) which is involved in cholesterol synthesis, cholesterol 7α-hydroxylase (7) which is involved in bile acid synthesis, as well as various enzymes involved in drug metabolism (8). We have examined the effect of food, fasting and adrenalectomy on the diurnal variation of Acid CEH activity in livers of rats. In this paper, we report a regular daily variation in the rate of cholesteryl ester hydrolysis in livers of rats.

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MATERIALS AND METHODS

Radiochemical

Cholesteryl [1-^{14}C] oleate (specific activity 58.6 mCi/m mole) was purchased from New England Nuclear Corp.

Animals

Young male Sprague-Dawley rats weighing about 150 g were used for all studies. Animals were offered food (Japan Crea Corp.) and water ad libitum and were housed in small groups in wire cages with a strict lighting schedule of 12 hr of light and 12 hr of darkness (light on at 06.00, off at 18.00). Animals were adapted to these conditions for at least one week prior to any experiment. Fasted rats were without food for 48 hr before sacrifice.

Adrenalectomy

Bilateral adrenalectomies were performed under ether anesthesia 5 days prior to the particular experiment. Adrenalectomized animals were maintained on 0.9% NaCl solution.

Preparation of Lysosomal Fractions and Measurement of Acid Cholesteryl Ester Hydrolase Activity

The methods of Brecher et al (9) were used for the preparation of lysosomal fractions from liver, spleen and brain and for the measurement of Acid CEH activity. Benzene solutions of cholesteryl oleate and cholesteryl [1-^{14}C] oleate were mixed and the benzene was evaporated under nitrogen. A saline solution with 0.5%

albumin was added and the mixture was sonicated 3 times for 10 sec. The standard incubation mixture usually contained 0.69 nmol of cholesteryl [1-^{14}C] oleate (0.04 μCi) in 0.15 M acetate buffer (pH 4.5) and 50 μg of lysosomal protein in a final volume 0.3 ml. The tubes were incubated at 37 C for 30 min. The reaction was terminated by addition of 3.0 ml of benzene/chloroform/methanol mixture (1:0.5: 1.2, v/v/v) containing unlabeled oleic acid (0.1 mM) as carrier. NaOH (0.6 ml of 0.3 M) was then added. The solution was mixed for 25 sec on a vortex mixer and centrifuged for 10 min at 3,000 rpm. The amount of liberated [1-^{14}C] oleate in the upper aqueous phase was determined by adding 0.5 ml aliquot to 10 ml of Aquasol 2 liquid scintillation mixture and counting the samples in an Aroka LSC 900 liquid scintillation counter.

RESULTS

Normal Diurnal Variation

Acid CEH activity, in livers of rats, was measured at 6 hourly intervals over a 24-hr period (Fig. 1). The Acid CEH activity exhibited a diurnal variation. Maximum specific activity of the Acid CEH was reached in the last of the dark period at 06.00 hours, and the activity then declined to a broad minimum at 18.00 hours. In the evening, the specific activity began to increase again. The amplitude of the variation as defined by the ratio of the maximum (06.00 hours) to the minimum rates (18.00 hours) was approximately 1.5. No variation in the enzyme activity in spleen and brain was observed (data not shown).

Effect of Restricted Feeding and Fasting with Normal Lighting

The importance of feeding habits rather than lighting in the diurnal rhythm of Acid CEH activity was demonstrated in rats housed under normal illumination conditions and offered food from 09.00 hours to 17.00 hours for 24 days prior to being killed. The maximum specific activity of Acid CEH occurred in the last part of the food period at 17.00 hours (data not shown). The result shows that the diurnal rhythm responded to the new feeding schedule.

Table 1 shows the effect of fasting on the Acid CEH activity at 06.00 hours and 18.00 hours. Fasting for two days abolished the diurnal rhythm and caused the Acid CEH activity to remain at a uniformly low level.

Effect of Adrenalectomy on Acid CEH Activity

Several investigations suggest that some diurnal rhythms may be controlled hormonally. We measured enzyme activities at 06.00 hours and 18.00 hours in livers of rats 5 days after bilateral adrenalectomy. As shown in Table 1, adrenalectomy appeared to have no effect on the diurnal rhythm of Acid CEH activity.

DISCUSSION

It is well known that hepatic 3-hydroxy-3-methylglutaryl-CoA reductase (6,10-12) and

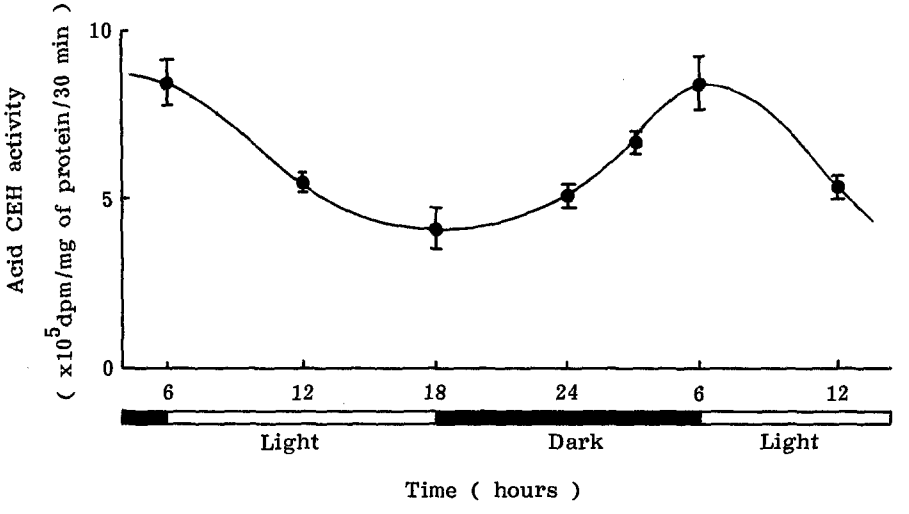

FIG. 1. Diurnal variation of Acid CEH activity in rat liver. Standard assay conditions were used as described in Materials and Methods. Values are given as mean ± SEM of 5 rats.

TABLE 1

Effect of Adrenalectomy and Fasting on the Diurnal Rhythm of Acid CEH Activity

Experiment	Treatment	Acid CEH activity ($\times 10^5$ dpm/mg of protein/30 min) 06.00 hr	18.00 hr
1	control	7.25 ± 0.24	4.56 ± 0.53[a]
	fasted	4.99 ± 0.32	4.19 ± 0.45[a]
1	control	6.60 ± 0.08	4.05 ± 0.01[a]
	adrenalectomized	4.41 ± 0.17	3.21 ± 0.36[a]
2	control	5.35 ± 0.15	3.72 ± 0.13[a]
	adrenalectomized	4.23 ± 0.80	2.73 ± 0.58[b]

Standard assay conditions were used as described in Materials and Methods. Values are given as mean ± SEM of 5 rats.

[a]Significantly different from 06.00 hours values ($P<0.01$).

[b]Significantly different from 06.00 hours values ($P<0.05$).

cholesterol 7α-hydroxylase (7,13,14) exhibit a diurnal rhythm. The experiments on the manner in which the diurnal rhythm in both enzymes is controlled have focused increasingly on the role of hormones (15-18).

The results in this paper show that when rats are kept under conditions of controlled lighting and feeding, a diurnal rhythm exists in lysosomal Acid CEH with maxima at 06.00 hours and minima at 18.00 hours. The rhythm of this enzyme activity is not affected by adrenalectomy. These data indicate that adrenal gland hormones are not involved in the variation of Acid CEH activity.

Whether feeding regulates the rhythm of Acid CEH activity in normal rats is an important question. We tested the effect of fasting on the Acid CEH activity and found that fasting indeed abolished the rhythm. Furthermore, the Acid CEH activity continued at the minimum level of the rhythm during the fasting. It is possible that the disappearance of the diurnal rhythm of the Acid CEH activity in fasting rats is not due to a general reduction in protein synthesis but rather to the restricted feeding in the dark period. However, it is not clear at present if the diurnal rhythm of Acid CEH activity results from changing the turnover rate of this enzyme.

Acid CEH was present in all tissues examined, and high activity was observed in spleen, lung and liver (4). The spleen and brain did not exhibit a diurnal variation of Acid CEH similar to that of the liver. These results suggest that the diurnal rhythm of Acid CEH may be characteristic of the liver, which is mainly concerned in the catabolism of cellular cholesteryl esters.

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Effects of High Fat Diets on the Activity of Palmitoyl-CoA Hydrolase in Rat Liver

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ABSTRACT

Palmitoyl-CoA hydrolase [EC 3.1.2.2.] activity in rat liver was found to be enhanced by high fat diets. Partially hydrogenated marine oil and high-erucic acid rapeseed oil diets produced a greater increase than a diet containing soybean oil.

With diets containing from 5 to 30% (w/w) of partially hydrogenated marine oil the increase in palmitoyl-CoA hydrolase activity was similar to the increase observed in peroxisomal β-oxidation activity (correlation coefficient r = 0.94). A positive correlation (r = 0.86) also was observed between the activity of palmitoyl-CoA hydrolase and previously determined levels of long-chain acyl-CoA.

The results presented may suggest a common "induction" mechanism for palmitoyl-CoA hydrolase and peroxisomal β-oxidation enzymes, possibly exerted through an increased cellular level of long-chain acyl-CoA.

Lipids 20:49-52, 1985.

INTRODUCTION

Palmitoyl-CoA hydrolase (EC 3.1.2.2.) catalyzes the hydrolysis of long-chain acyl-CoA thioesters to CoASH and fatty acids. In rat liver, the subcellular distribution and activity changes due to hypolipidemic drug administration have been studied extensively (1-5). These studies have revealed that the palmitoyl-CoA hydrolase has a trimodal localization: to the mitochondrial and microsomal fractions (3-6) and to a purified fraction enriched in peroxisomes (4-7). However, the functional roles of these enzyme activities still are not completely clarified.

Following administration to rats of peroxisomal proliferators unrelated in chemical structure, palmitoyl-CoA hydrolase as well as peroxisomal β-oxidation increased (2,3,5). Peroxisomal β-oxidation activity also is found to be elevated in different physiological conditions, such as during starvation (8,9) and high fat feeding (8-11).

In the present work we have compared the effect of some high fat diets on rat liver palmitoyl-CoA hydrolase and peroxisomal β-oxidation. The data presented show that the variations in the two enzyme activities are well correlated, which may indicate a common "induction" mechanism. These findings are discussed in relation to the linearity recently observed between the level of long-chain acyl-CoA in rat liver and the activity of peroxisomal β-oxidation (12,13).

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EXPERIMENTAL

Materials

Dietary oils were obtained from DeNoFa and Lilleborg Fabriker A/S, Fredrikstad, Norway, except for the rapeseed oil, which was supplied by AB Karlshamns Oljefabriker, Karlshamn, Sweden. The fatty acid compositions are given in (8). [1-^{14}C] palmitoyl-CoA was purchased from New England Nuclear, Boston, Massachusetts, USA. Other chemicals were commercial products of high purity, mostly from Sigma Chemical Co., St. Louis, Missouri, USA.

Animals and Diets

Weanling male rats of the Wistar strain (body weight approx. 60 g) were purchased from Møllegaard breeding laboratory, Ejby, Denmark. Semisynthetic diets were prepared as described previously (14), with the compositions (in weight percentage of total) as follows: sucrose, 20.0%; corn starch, 52.8%, minus weight percentage of dietary oil; casein (with 2% methionine), 20.0%; cellulose 1.0%; vitamin mixture, 2.2%; salt mixture, 4.0%; dietary oil, according to tables. The rats were fed a standard pelleted diet for 5 days, and then given the semisynthetic diets for 3 weeks. They were housed in grid-bottomed cages and had free access to food and water. At the end of the experimental period the rats were killed by decapitation, the livers were removed and immediately chilled on ice and weighed. Preparation of the 10% liver homogenate was performed as earlier described (8).

TABLE 1

Effects of Different Dietary Oils on Rat Liver Palmitoyl-CoA Hydrolase Activity

Diet	Palmitoyl-CoA hydrolase					
	μmole/min/ whole liver	% of SO 5	μmole/min/ g · liver	% of SO 5	nmol/min/ mg prot	% of SO 5
SO 5	11.9 ± 3.2	100	1.16 ± 0.36	100	10.0 ± 1.0	100
SO15	16.6 ± 2.8	140	1.52 ± 0.35	130	12.9 ± 1.5	130
RO 15	20.9 ± 4.9[b]	175	1.80 ± 0.48[a]	155	17.0 ± 2.0[b]	170
PHMO 15	26.1 ± 4.3[b]	220	2.13 ± 0.18[b]	185	20.8 ± 2.5[b]	210

The values are means ± S.D. for 4 animals in each group. SO 5 and SO 15 = diets containing 5 and 15% (by weight) of soybean oil, respectively. RO 15 = diet containing 15% of a high-erucic rapeseed oil (about 45% $C_{22:1}$). PHMO 15 = diet containing 15% of partially hydrogenated marine oil (about 15% $C_{22:1}$). The palmitoyl-CoA hydrolase activity was measured at 30 C.

[a]Values significantly different from those of the SO 5 group, $0.05 > P > 0.01$.

[b]Values significantly different from those of the SO 5 group, $0.01 > P$.

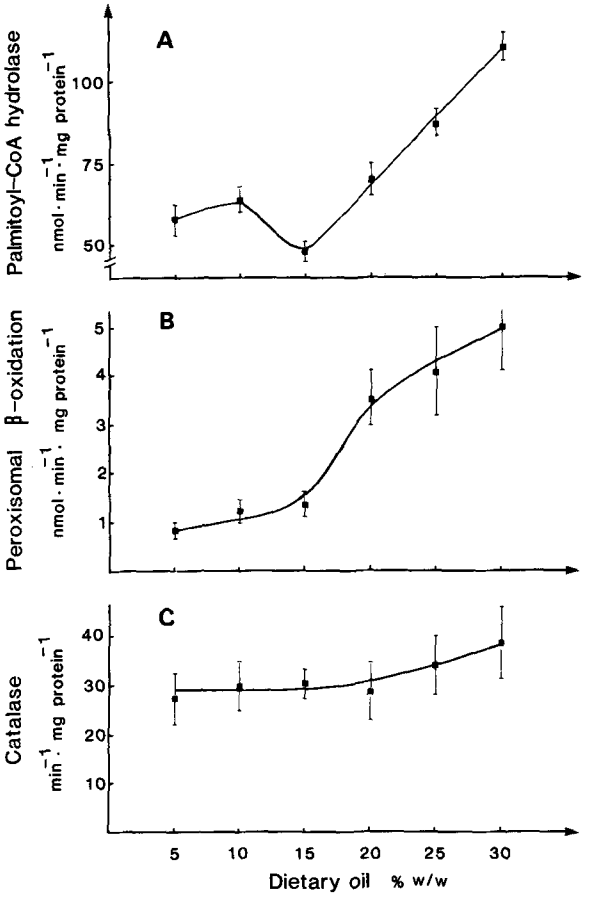

FIG. 1. Effect of increasing amounts of partially hydrogenated marine oil in the diet on some enzyme activities determined in the 10% liver homogenate. Values represent means ± S.D. of 4 rats. A, Palmitoyl-CoA hydrolase (at 35 C); B, peroxisomal β-oxidation; C, catalase.

Enzyme and Protein Assays

Palmitoyl-CoA hydrolase (EC 3.1.2.2.), catalase (EC 1.11.1.6.) and CN^--insensitive, palmitoyl-CoA dependent NADH production (peroxisomal β-oxidation) were determined as described in (6), (1,3) and (15), respectively. SDS-polyacrylamide gel electrophoresis (9% acrylamide) was carried out as described elsewhere (3), and protein was determined using the Folin-Ciocalteus reagent (16) with bovine serum albumin as standard.

Statistical Analysis

Dunnett's multiple comparison test (17) was used to evaluate the significance of differences between population means; $P > 0.05$ was taken to be statistically non-significant.

RESULTS AND DISCUSSION

The palmitoyl-CoA activities observed in rat liver homogenates after 3 weeks on different high fat diets are shown in Table 1. A low-fat diet containing 5% by weight of soybean oil has been included for reference. Significantly higher activities (170-220%) were observed in the rats fed rapeseed oil or partially hydrogenated marine oil than in this low-fat control. Also, the rats fed a high fat diet containing soybean oil showed a tendency toward higher values (130-140%). The difference, however, was not statistically significant. These results correspond well with the 1.4- to 2.4-fold increase in peroxisomal β-oxidation activity previously observed employing similar diets (8,10,11), suggesting that with respect to high fat diets the effects on

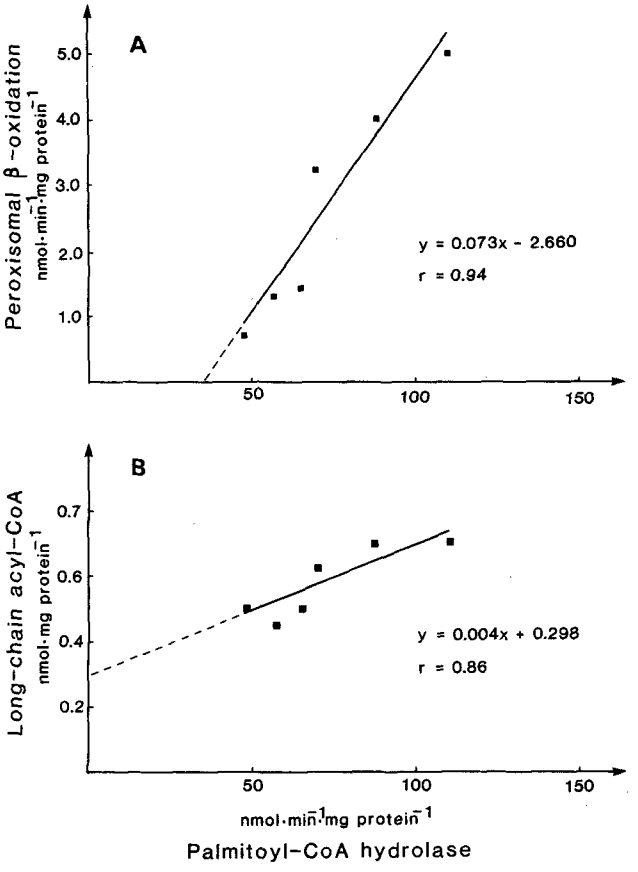

FIG. 2. Correlation between palmitoyl-CoA hydrolase activities and (A) peroxisomal β-oxidation activity ($r=0.94$; $0.01>P>0\ 005$), and (B) long-chain acyl-CoA levels ($r=0.86$; $0.05>P>0.02$). The values of palmitoyl-CoA hydrolase and peroxisomal β-oxidation activity are taken from Figure 1 A and B. The values of long-chain acyl-CoA levels are taken from (13).

palmitoyl-CoA hydrolase and peroxisomal β-oxidation activity are very similar.

To investigate this further we compared the effects of diets containing various amounts of partially hydrogenated marine oil on the two enzyme systems, since with such diets a sigmoidal dose-response relationship has been observed with regard to the activity of peroxisomal β-oxidation in peroxisome-enriched fractions from rat liver (8). This sigmoidal dose-dependency also is seen in the 10% homogenate from rat liver, as shown in Figure 1B. The marked increase with 20% or more of partially hydrogenated marine oil in the diets correlated well with an increase observed in a band corresponding to a molecular weight of 80,000 on SDS-polyacrylamide gel electrophoresis (results not shown). This band (PPA-80), which is immunologically identical to the peroxisomal enoyl-CoA hydratase (18), has been found to account for a large fraction of the increase of peroxisomal protein in liver of rats treated with peroxisome proliferators (18,19). The observed increase indicated, in agreement with the results obtained by Ishii et al. (9), that the increase in the activity of peroxisomal β-oxidation after high fat feeding is due to enzyme induction.

Also the activity of the palmitoyl-CoA hydrolase in the 10% homogenate revealed a strong dose-dependency as shown in Figure 1A. As with peroxisomal β-oxidation, the palmitoyl-CoA hydrolase activity increases markedly with 20% or more of partially hydrogenated marine oil in the diet. At the highest dose (30%), the enzyme activity is enhanced by a factor of about 1.7. The catalase activity, on the other hand, is affected only slightly by the dose of partially hydrogenated marine oil in the diet (Fig. 1C).

Thus, palmitoyl-CoA hydrolase activity in rat liver seems to be regulated by the dietary level of fat, and in a way similar to that observed for peroxisomal β-oxidation. In fact, when comparing the activities found at different dietary levels of partially hydrogenated marine oil, a correlation coefficient of 0.94 is obtained (Fig. 2A). These observations might indicate a common regulatory mechanism. The cellular level of long-chain acyl-CoA is found to correlate well with the activity of peroxisomal β-oxidation after feeding rats diets with various amounts and different types of fat (13). Comparing the activity of palmitoyl-CoA hydrolase found in the present work and the long-chain acyl-CoA values reported in an earlier study using the same diets (13) revealed a linear correlation as shown in Fig. 2B, with a correlation coefficient of 0.86. Furthermore, the level of cellular long-chain acyl-CoA has been observed to correlate well with the activity of palmitoyl-CoA hydrolase in animals where this activity has been increased by the administration of the hypolipidemic drugs clofibrate and tiadenol (Berge, unpublished observations). A regulatory action of long-chain acyl-CoA in gene expression has been postulated in *Candida Lipolytica* (20). Long-chain acyl-CoA is the substrate of both the palmitoyl-CoA hydrolase and the peroxisomal β-oxidation enzyme system, and a regulation by substrate induction thus may be suggested.

ACKNOWLEDGMENT

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Effect of Type of Diet and Feeding Status on Modulation of Hepatic HMG-CoA Reductase in Rats

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ABSTRACT

The effect of diet type and feeding status on hepatic HMG-CoA reductase (HMGR) [mevalonate: $NADP^+$ oxidoreductase (acylating CoA); EC 1.1.1.34] was studied in rats. Animals fed a ground, commercial, stock diet exhibited higher expressed and total activities of HMGR in the fed state than animals fed a semi-purified diet. The differences did not appear in meal-trained animals when measured before the onset of the meal after a 22-hr fast. When expressed activity was taken as a per cent of total activity, fed animals from both diet groups used about 10% of their available activity. When animals on commercial diets were fasted, 20% of the activity was expressed. Fasted animals on the semi-purified diet also increased the per cent of expressed reductase activity, but this increase was not as great (13.3%).

These data suggest that, in the rat, regulation of cholesterol synthesis in response to decreased total HMGR during fasting and increased levels after a meal results from alterations in the percentage of enzyme which is expressed. The semi-purified diet used here resulted in consistently lower levels of HMG-CoA reductase activity than the commercial diet regardless of feeding pattern.

Lipids 20:53-55, 1985.

INTRODUCTION

The rate-limiting step in the biosynthesis of cholesterol is catalyzed by β-hydroxymethylglutaryl-CoA reductase (HMGR;1). Ingebritsen et al. (2) proposed a scheme for the modulation of HMGR activity by reversible phosphorylation of the enzyme. Subsequently they demonstrated that the degree of phosphorylation and the activity of HMGR in isolated rat hepatocytes could be manipulated by the addition of glucagon or insulin to the incubation medium (3).

Whether HMGR is modulated by such a mechanism in vivo is controversial. Brown et al. (4) reported that the modulation of rat microsomal HMGR was not changed when rats were subjected to light cycling, fasting, stress, cholesterol feeding or cholestyramine. However, Arebalo et al. (5) found that the feeding of a cholesterol-containing meal to rats resulted in an inhibition of liver microsomal HMGR within one hr, and that this inhibition could be reversed by incubation of the microsomes with phosphatase.

The type of diet fed during such experiments may itself be a factor in the regulation of HMGR activity. To test this possibility, rat liver microsomal HMGR activity was measured under two conditions, the fasted state in which animals were known to be physiologically adapted to fasting, and the fed state at the midpoint of the dark period of the light cycle, measuring activities with respect to diet at times when HMGR activity is known to be at its lowest and highest levels, respectively. Two types of diet which commonly are used as control diets were investigated under these conditions: a commercial, natural ingredient diet (Wayne Lab Blox) and a semi-purified diet. The results indicate that while the amount of HMGR varies with the type of diet, modulation of the enzyme seems to be associated with the feeding status.

MATERIALS AND METHODS

Animals were housed under conditions of constant temperature and humidity (72 ± 2 F; 80%) with 12 hr dark and 12 hr light. Animals had access to deionized water ad libitum. Male Wistar rats (Harlan-Sprague-Dawley, Indianapolis, Indiana) weighing 80-116 g were fed either ground, Wayne Lab-Blox (minimally, diet contained 24% crude protein, 4.0% crude fat and 4.5% crude fiber) (Allied Mills, Inc., Chicago, Illinois) or a semi-purified (SP) diet (diet contained 50% glucose-hydrate, 20% casein [high nitrogen], 10% corn oil, 15% cellulose, 4% A.I.N. mineral mix and 1% A.I.N. vitamin mix) ad libitum for 3 days. Animals then were divided into 4 groups of 10. Two groups gradually were adapted to meal feeding which consisted of feeding commercial or SP diet for 2 hr daily at the onset of the dark period. The other two groups remained on an ad libitum regimen.

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After two weeks animals fed ad libitum were killed at the mid-point of the dark period and meal-fed animals were killed 30 min before the onset of the meal. No significant differences in body weight were observed between groups within feeding patterns. Rats fed ad libitum weighed significantly more than those which were meal-fed either diet.

Isotopic [3-^{14}C]-hydroxymethylglutaric acid (56.6 Ci/mol) and [5-^{3}H]-mevalonolactone (9.3 Ci/mol) were obtained from Amersham Corporation (Arlington Heights, Illinois). Coenzyme A and hydroxymethylglutaryl-CoA were from the Sigma Chemical Co. (St. Louis, Missouri). Synthesis of [3-^{14}C]-hydroxymethylglutaryl-Coenzyme A was accomplished by the method of Williamson and Rodwell (6).

Animals were killed by decapitation and samples of plasma and liver taken for cholesterol and triglyceride determination. Cholesterol was measured by the method of Rudel and Morris (7); triglycerides were analyzed by the method of van Handel and Zilversmit (8). Livers were excised, rinsed in ice cold 0.25M sucrose and 1-gram portions homogenized in 0.25M sucrose containing 5mM dithiothreitol with and without fluoride (F^-). Homogenates were centrifuged at 500 x g for 5 min, then 10,000 x g for 10 min. Microsomes were isolated by centrifuging the supernatant at 105,000 x g for 60 min. HMGR activity was assayed in the presence and absence of 50mM F^- by the method of Nordstrom et al. (9). Analysis of variance was carried out for each variable and, where appropriate, means were compared using least significant difference (10).

RESULTS AND DISCUSSION

The effects of the diet and feeding status on serum and liver lipids are shown in Table 1. The SP diet yielded higher serum cholesterol levels when animals were fed ad libitum or fasted and higher liver cholesterol levels with ad libitum feeding only. Serum triglycerides were affected by feeding status only, with higher values obtained in the fed animals. Liver triglycerides were elevated in SP animals, with the highest levels occurring in fed animals. This interaction between diet and feeding status was statistically significant.

Activities of HMGR measured in the presence (R_a) and absence (R_t) of 50mM F^- are presented in Table 2. Fasted animals from both diet groups exhibited similarly low R_a values. While R_a values for fed animals on the SP diet remained low, R_a was nearly doubled in fed animals on the commercial diet. Similarly, fasted animals had much lower R_t levels than fed animals when either diet was used, although the difference was significant only in the commercial diet animals ($p<0.05$). Commercial diet,

TABLE 1

Serum and Liver Cholesterol and Triglycerides (Means ± S.E.M.)

	Commercial diet		Semipurified diet	
	Fed	Fasted	Fed	Fasted
Serum cholesterol (mg/dl)	52.4 ± 3.2 ^{a}	54.9 ± 2.5 ^{a}	68.5 ± 3.7 ^{b}	67.6 ± 4.3 ^{b}
Liver cholesterol (mg/g)	2.17 ± 0.05^a	2.32 ± 0.07^a	3.99 ± 0.31^b	3.74 ± 0.37
Serum triglycerides (mg/dl)	123.9 ± 6.3 ^{a}	68.2 ± 4.6 ^{b}	146.1 ± 19.7	58.5 ± 5.3 ^{b}
Liver triglycerides (mg/g)	6.36 ± 0.66^a	3.28 ± 0.47^a	23.13 ± 3.60^b	15.20 ± 3.44^c

Means not sharing a common superscript (within each parameter) are significantly different (1sd, $p<0.05$).

TABLE 2

HMG-CoA Reductase Activities (Mean ± S.E.M.)

	Commercial diet		Semipurified diet	
	Fed	Fasted	Fed	Fasted
	pmoles · min^{-1} · mg^{-1}			
Expressed activity (R_a)	111.8 ± 14.4^a	64.2 ± 18.4^b	55.5 ± 7.9^b	48.3 ± 19.2^b
Total activity (R_t)	1188.0 ± 134.3^a	329.9 ± 84.1^b	580.8 ± 65.8^b	303.2 ± 94.9^b
R_a/R_t	0.102 ± 0.017	0.200 ± 0.053	0.098 ± .012	0.133 ± 0.020

Means not sharing a common superscript (within parameters) are significantly different (1sd, $p<0.05$).

ad libitum fed animals had a significantly higher R_t than the ad libitum, SP animals ($p<0.05$). Although the R_t value in animals fed the SP diet ad libitum increased, the difference was not statistically significant. The R_t value for ad libitum fed animals on commercial diet showed nearly a 10-fold increase over R_t values, a statistically significant change (p 0.05). The R_a/R_t values in both diet groups rose when the animals were fasted, being almost twice as high in the commercial diet group and 36% higher in the SP group. R_a/R_t value is not an independent measure and therefore cannot be tested for statistical significance.

The serum and liver lipid data are consistent with those of other workers who have shown reduced cholesterol levels in animals fed a commercial diet and increased liver triglycerides in animals fed a semi-purified diet (11). The values of HMGR activity are consistent with the reduced cholesterol levels in response to the commercial diet in that both the active portion (R_a) and total amount of enzyme (R_t) were greater in animals fed the commercial diet responding to increased demand for sterol as a result of increased fecal steroid output (12). These data for R_t are in agreement with those of Reiser et al. (13) and extend our knowledge of the amount and active portion of HMGR present and increasing the portion of enzyme that was active. These data suggest that meal pattern and diet composition alter the activity of HMGR and that control of these nutritional variables is an important consideration when working with this enzyme.

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[Received January 30, 1984]

Comments on Lipid Contaminants in Commercial Lipases

Sir:

The presence of contaminating lipids in pancreatic lipase preparations reported in Lipid Contaminants in Commercial Lipases, *Lipids* 19:302-3, 1984 (1) and their removal by extraction has been known for many years. Litchfield (2) lists four papers in which solvent extraction was used to remove contaminating lipids from commercial pancreatic lipase preparations; one of these was reference 3 in the paper above. We reported in 1964 (3) that it was necessary to extract the pancreatic lipase preparation we were using. We have since routinely extracted crude preparations four times with diethyl ether. Employment of a proper control (minus substrate) with internal standards will enable the investigator to correct for lipid contaminants. We have done this when the enzyme was scarce and we did not know if solvent extraction would cause loss of enzyme activity. Prior extraction, when possible, is obviously much simpler.

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Letter to the Editor

Response to Comments made by Dr. R.G. Jensen on Lipid Contaminants in Commercial Lipases, *Lipids* 19:302-303, 1984.

Sir:

We agree that prior extraction of lipase has been known for many years, as pointed out by Dr. R.G. Jensen. We are sorry for not mentioning all the references indicated by Dr. Jensen. Prior extraction of lipase is necessary for lipolysis of wax esters (1) for the following reasons: Our present letter (2) described prior removal of lipids in the context of the lipolysis of wax esters, where fatty acid analysis is essential. Because commercial lipase contains mainly free fatty acids as contaminants (2), it affects fatty acid composition of the wax severely. Use of a control with an internal standard would be erroneous when components in the sample are present in low levels compared to those in the contaminants. This is obvious because, for utilizing wax esterase activity of lipase, a substantially high enzyme to substrate ratio must be used (1). Obviously prior extraction is much simpler, as indicated by Dr. Jensen, and we have observed that activity is not altered appreciably even after extraction (2). Our communication also reported the composition of the lipid contaminants of two commercial lipases.

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[Received June 20, 1984]

1985 AOCS Annual Meeting
Technical Sessions on Lipids and Related Topics

The following sessions have been announced as part of the tentative technical program for the 76th Annual Meeting of the American Oil Chemists' Society to be held May 5-9, 1985, in the Franklin Plaza Hotel in Philadelphia, PA. Further details, registration forms and housing reservation forms are available from: Meetings Coordinator, AOCS, 508 S. Sixth St., Champaign, IL 61820 USA.

Neurochemical Aspects of Lipid Metabolism: Selected Topics

Chairperson: S.L. Miller, The Wistar Institute, Philadelphia, PA

DIFFERENT FUNCTION OF BRAIN PHOSPHOLIPIDS–STRUCTURAL AND SECOND MESSENGER ROLES
P. Morell, University of North Carolina, Chapel Hill, NC

GANGLIOSIDES AND THEIR ROLE IN NERVOUS TISSUE
R. Yu, Yale University, New Haven, CT

EFFECTS OF ISCHEMIA AND BRAIN STIMULATION ON BRAIN LIPID METABOLISM
N. Bazan, Lousiana State University Eye Center

SPHINGOLIPIDOSIS
K. Suzuki, Albert Einstein College of Medicine, New York, NY

ESSENTIAL FATTY ACID DEFICIENCY–EFFECTS ON BRAIN LIPIDS
S.L. Miller, The Wistar Institute, Philadelphia, PA

High Performance Liquid Chromatography in the Analysis of Lipids I

Chairperson: E.G Perkins, University of Illinois, Urbana, IL

FRACTIONATION AND ANALYSIS OF LIPID CLASSES, FATS AND OILS BY HPLC VIA A FLAME IONIZATION DETECTOR
O.S. Privett, W.L. Erdahl and F.C. Phillips, Hormel Institute, University of Minnesota, Austin, MN

HPLC OF TRIGLYCERIDES USING GRADIENT ELUTION
B. Hersloef, G. Kindmark, C. Thoerngren, KabiVitrum AB, Stockholm, Sweden.

REVERSE PHASE HPLC OF PHOSPHOLIPIDS
N. Sotirhos, B. Hersloef, C. Thoerngren, KabiVitrum AB, Stockholm, Sweden

HPLC OF TRIGLYCERIDES SYSTEM AND SOLVENT EFFECTS
E.G. Perkins and David Hendren, University of Illinois, Urbana, IL

HIGH PERFORMANCE GEL PERMEATION CHROMATOGRAPHY OF METHYL ESTERS, MONO-, DI- AND TRIGLYCERIDES MIXTURES
C.N. Christopoulou and E.G. Perkins, University of Illinois, Urbana, IL

Specialty Lipids and Their Biofunctionality I

Chairpersons: V.K. Babayan, Harvard Medical School, Boston, MA and J. Kabara, Michigan State University, East Lansing, MI

MEDIUM CHAIN TRIGLYCERIDES AND STRUCTURAL LIPIDS
V.K. Babayan, Harvard Medical School, Boston, MA

ABSORPTION OF SAFFLOWER OIL AND STRUCTURED LIPID PREPARATIONS IN PATIENTS WITH CYSTIC FIBROSIS
V.S. Hubbard, National Institutes of Health, Bethesda, MD, and M.C. McKenna, University of Maryland School of Medicine, Baltimore, MD

ICOSANOID SYNTHESIS BY PLATELETS OF CHILDREN WITH CHOLESTATIC DISEASE
O. Amédée-Manesme, l'Hopital de Bicêtre, Bicêtre, France, and J. Dupont,* Iowa State University, Ames, IA

INFLUENCE OF MEDIUM CHAIN TRIGLYCERIDES ON RAT MAMMARY TUMOR DEVELOPMENT
L.A. Cohen, Naylor Dana Institute of Disease Prevention, Valhalla, NY, and D.O. Thompson, State University of New York, Purchase, NY

MEDIUM CHAIN TRIGLYCERIDES AND STRUCTURED LIPIDS AS UNIQUE NON-ENERGY SOURCES IN HYPERALIMENTATION
E.A. Mascioli, New England Deaconess Hospital, Boston, MA, and V.K. Babayan and G.L. Blackburn, Harvard Medical School, Boston, MA

Lipid Metabolism in Disease

Chairperson: D. Kritchevsky, The Wistar Institute, Philadelphia, PA

DIETARY PROTEIN AND ATHEROSCLEROSIS
D. Kritchevsky, S.A. Tepper and D.M. Klurfeld, The Wistar Institute, Philadelphia, PA

DIETARY PROTEIN EFFECTS ON GALLSTONE FORMATION IN HAMSTERS
D.M. Klurfeld and D. Kritchevsky, The Wistar Institute, Philadelphia, PA

INABILITY OF SKIN ENZYME PREPARATIONS TO BIOSYNTHESIZE ARACHIDONIC ACID FROM LINOLEIC ACID
R. Chapkin and V.A. Ziboh, University of California, Davis, CA (Honored Student Presentation)

ISOPRENE: BIOSYNTHESIS AND ROLE IN POLYISOPRENOID METABOLISM
E.S. Deneris, University of California, Los Angeles, CA (Honored Student Presentation)

STUDIES ON THE METABOLISM OF MALONDIALDEHYDE
H.H. Draper, L.G. McGirr, M. Hadley and L. Polensek, University of Guelph, Guelph, Ontario, Canada

Thermal-Oxidative Effects on Lipids

Chairperson: E.H. Hammond, Iowa State University, Ames, IA

AN HPLC METHOD FOR ANALYZING HIGH MOLECULAR WEIGHT COMPOUNDS FORMED IN HEATED OILS
P.J. White and Y-C. Wang, Iowa State University, Ames, IA

DIMER ISOLATION AND CHARACTERIZATION IN THERMALLY OXIDIZED FATS
C.N. Christopoulou and E.G. Perkins, University of Illinois, Urbana, IL

COMPARISON OF THE CYCLIC FATTY ACID MONOMERS FORMED DURING THE HEAT TREATMENT OF VEGETABLE OILS
J.L. Sebedio and J. Prevost, I.N.R.A., Dijon Cedex, France, and O. Morin, ITERG, Pessac, France

IDENTIFICATION IN LIVER LIPIDS OF RATS FED A HIGH LINOLENIC ACID HEATED OIL OF A 20:5 FATTY ACID HAVING A *trans* ETHYLENIC BOND
A. Piconneaux, A. Grandgirard and J.L. Sebedio, I.N.R.A., Dijon Cedex, France

ENZYMATIC HYDROLYSIS OF THERMALLY OXIDIZED CANOLA OIL
J.C. Alexander and H. Yoshida, University of Guelph, Guelph, Ontario, Canada

THERMAL OXIDATION OF BUTTERFAT AND BUTTERFAT FRACTION IN COMPARISON TO SELECTED VEGETABLE OILS
D.B. Kupranycz, M.A. Amer and B.E. Baker, McGill University, Ste Anne de Bellevue, Quebec, Canada

RETARDATION OF RANCIDITY IN DEEP-FRIED INSTANT NOODLE (RAMYON)
K.L. Rho, P.A. Seib and D.S. Chung, Kansas State University, Manhattan, KS, and O.K. Chung, USDA Grain Marketing Research Laboratories and Kansas State University, Manhattan, KS (Honored Student Presentation)

THE REPROCESSING AND REUSE OF SPENT RESTAURANT GREASE AND THE ENVIRONMENTAL CONTROL PROCEDURES NECESSARY
B.F. Osborne, West Coast Reduction Ltd., Vancouver, British Columbia, Canada

A TECHNIQUE FOR MONITORING THE QUALITY OF USED FRYING OILS
P-F. Wu and W.W. Nawar, University of Massachusetts, Amherst, MA

HPLC in the Analysis of Lipids II

Chairperson: E.G. Perkins, University of Illinois, Urbana, IL

USE OF THE LIGHT SCATTERING DETECTOR IN ANALYSIS OF LIPIDS
A. Prevot, Institute des Corps Gras, Pessac, France, J.L. Perrin, ITERG, and H. Stolywho and G. Guiochon, Ecole Polytechnique, Palaiseau, France

HPLC OF FATTY NITROGEN DERIVATIVES OR FROM ON THE HOOF TO ON THE ROAD
G. Szajer and L. Yodual, Akzo Chemie America, McCook, IL

IDENTIFICATION OF FATTY ACID ESTERS OF CHLOROPROPANEDIOL IN MILK FATS BY LC/MS
J. Cerbulis, O.W. Parks and H.M. Farrell Jr., USDA Eastern Regional Research Center, Philadelphia, PA, and A. Kuksis, L. Marai and J.J. Myher, University of Toronto, Toronto, Ontario, Canada

A NEW FLAME IONIZATION DETECTOR FOR THE LIQUID CHROMATOGRAPHY OF OILS, ETC.
J.B. Dixon, Tracor Instruments Austin Inc., Austin, TX

OVERPRESSURE LAYER CHROMATOGRAPHY
J.M. Newman, Newman-Howells Associates Ltd., Winchester, England

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E.N. Christiansen, University of Oslo, Oslo, Norway, and J. Norseth and M.S. Thomassen, Norwegian Food Research Institute

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F.J. Bunick and W.W. Nawar, University of Massachusetts, Amherst, MA

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L-B. Hau, National Taiwan University, and W.W. Nawar, University of Massachusetts, Amherst, MA

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Chairpersons: R.G. Jensen, University of Connecticut, Storrs, CT, and J. Bitman, USDA BARC-E, Beltsville, MD

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J. Bitman and D.L. Wood, U.S. Department of Agriculture, Beltsville, MD, and N.R. Mehta, P. Hamosh and M. Hamosh, Georgetown University Medical Center, Washington, DC

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M.T. Clandinin, University of Alberta, Edmonton, Alberta, Canada, and J.E. Chappell, University of Toronto, Toronto, Ontario, Canada

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B.B. Teter, L.M. Neira, M. Keeney and J. Sampugna, University of Maryland, College Park, MD

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K.E. Hundrieser, R.M. Clark and R.G. Jensen, University of Connecticut, Storrs, CT

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C.J. Lammi-Keefe, F.M. Verda, A.M. Ferris, R.M. Clark and R.G. Jensen, University of Connecticut, Storrs, CT

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K.K. Carroll and L.M. Braden, University of Western Ontario, London, Ontario, Canada

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G.W. Patterson, G.P. Fenner and P.M. Koines, University of Maryland, College Park, MD

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P. Benveniste, Institut de Botanique, Strasbourg Cédex, France; A. Rahier, M. Taton, P. Nave, A.S. Narula and L. Cattel

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M.T. Clandinin, University of Alberta, Edmonton, Alberta, Canada; P.J.H. Jones, University of Toronto, Toronto, Ontario, Canada, and P.B. Pencharz, The Hospital for Sick Children, Toronto, Ontario, Canada

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T.J. Bach, University of Karlsruhe, Karlsruhe, West Germany

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J.C. Fogleman, University of Denver, Denver, CO, and S.M. Duperret and H.W. Kircher, University of Arizona

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C.E. Martin, Rutgers University, New Brunswick, NJ

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W.R. Nes, W.J. Pinto and I.C. Dhanuka, Drexel University, Philadelphia, PA

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Chairperson: D.P. Schwartz, USDA Eastern Regional Research Center, Philadelphia, PA

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STEREOSPECIFIC ANALYSIS OF SYNTHETIC AND NATURALLY OCCURRING FATTY ACID ESTERS OF CHLOROPROPANEDIOL

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AN IMPROVED METHOD FOR OBTAINING AND QUANTITATING THE UNSAPONIFIABLE MATTER OF FATS AND OILS

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RAPID ESTIMATION OF GLUCOSINOLATES BY GAS LIQUID CHROMATOGRAPHY OF CARBONYL SULFIDE

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A SIMPLE METHOD FOR QUANTITATIVE ISOLATION OF FREE FATTY ACIDS FROM FATS AND OILS

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Fatty Acid Composition of Phospholipids and Neutral Lipids during Embryonic and Early Larval Development in Atlantic Herring (*Clupea harengus*, L.)

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ABSTRACT

The fatty acid compositions of total polar and total neutral lipids of Atlantic herring eggs and larvae were determined immediately before fertilization, after fertilization and at various times during subsequent embryonic and early larval development. Within 3 hr after fertilization the percentage of total PUFA in neutral lipid decreased from 33% to 20%, with a reciprocal increase in monoenes. Thereafter the percentage of PUFA in the neutral lipids increased progressively, attaining the original level in ripe eggs by the time of yolk sac absorption. During the larval stages the percentage of PUFA continued to increase in the neutral lipid, reaching almost 44% of the total by day 32 after fertilization, although it was reduced to 32% by day 36. The percentage of monoenes in the neutral lipid displayed a progressive decrease during the whole period of development from 3 hr after fertilization. Throughout all the developmental periods the fatty acid composition of total polar lipids remained essentially constant. The polar lipids of the yolk sac displayed virtually the same fatty acid composition as the larval bodies, but the neutral lipids of the yolk sac were low in PUFA compared to the larval bodies. The results are discussed with reference to changes in lipid class composition during development. The conservation of high levels of PUFA in lipids during embryogenesis and early larval development reflects the importance of these fatty acids during development.
Lipids 20:69-74, 1985.

INTRODUCTION

We have studied changes in the lipid class composition during embryonic and early larval development in Atlantic herring (1), a species in which phospholipid accounts for almost 70% of the total lipid in the ripe egg (2). The results showed that there was a net consumption of phospholipid during the period of development up to the stage of yolk sac absorption. The decrease in phospholipid was due entirely to a net consumption of the major phospholipid class, phosphatidylcholine (PC), which initially accounted for almost 58% of the total lipid. Substantial changes in the composition of the major neutral lipids also occurred during this period.

The lipids of marine teleost eggs are rich in polyunsaturated fatty acids (PUFA) (2-7). Recently we noted that, in the eggs of several species including herring, almost 50% of the fatty acids present in phospholipids were polyunsaturated and 94% of these were (n-3) isomers (2). In neutral lipids PUFA accounted for 37% of the total fatty acids and were again predominantly (n-3) isomers. These results implied that the developing embryo and emerging larva has a requirement for high levels of (n-3) PUFA, perhaps greater than previously recognized (8).

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In the present paper we describe changes in the fatty acid compositions of the total phospholipid and total neutral lipid fractions that occur during embryonic and early larval development in the Atlantic herring. The results are discussed with reference to the changes in the lipid class composition during this period, described in the associated paper (1), and in relation to possible different roles of the various fatty acids.

MATERIALS AND METHODS

Eggs and Larvae

Details of the eggs and larvae and the incubation, rearing, sampling and storage procedures used were described earlier (1).

Analytical

Total lipid was extracted from the samples by the method of Folch et al. (9) and stored in chloroform/methanol (2:1, v/v) containing 0.05% butylated hydroxytoluene at -20 C between analytical procedures.

Fatty Acid Analyses

Polar and neutral lipids were separated by thin layer chromatography on silica gel 60 plates using hexane/diethyl ether/acetic acid (85:15:1,

v/v/v) as solvent. Neutral lipids were eluted with chloroform/methanol (2:1, v/v) and polar lipids were eluted with chloroform/methanol/distilled water (5:5:1, v/v/v). All solvents were supplemented with 0.05% butylated hydroxytoluene (BHT). Fatty acid methyl esters were prepared from neutral and polar lipids by acid-catalyzed transmethylation (10) followed by purification by thin layer chromatography. Samples were dissolved finally in hexane containing BHT. Analyses of the methyl esters were performed on a Packard 429 gas chromatograph (Packard Instruments Inc., Caversham, U.K.) equipped with a CP Wax 51 fused capillary column (50 m × 0.34 mm i.d.) (Chrompack, U.K., Ltd., London) and an on-column injection system (Scientific Glass Engineering [U.K.] Ltd., Milton Keynes) using helium as a carrier gas and a thermal gradient from 50 C to 225 C. Individual methyl esters were identified and quantified as described previously (2).

RESULTS AND DISCUSSION

Total neutral lipids from ripe eggs contained approximately equal amounts of each of the 3 major groups of fatty acids, saturated (31%), monounsaturated (34%) and PUFA (33%) (Table 1). Within 3 hr (0.1 day) of fertilization there was a marked increase in the relative percentage of monoenes with corresponding decreases in the percentages of saturates and, in particular, PUFA (Fig. 1). These changes were due mainly to a large increase in 18:1(n-9) and a large decrease in 22:6(n-3) together with a small decrease in 16:0. However, in contrast to the decreasing percentages of C_{20} and C_{22} PUFA, the C_{18} PUFA increased over this short period (Table 1). Overall, the changes in PUFA composition resulted in a marked decrease in the (n-3)/(n-6) ratio from 14.7 to 5.5 (Table 1). In the preceding paper we reported that within 3 hr of fertilization there was a decrease in the lipid content of fertilized herring eggs and the percentage of neutral lipid decreased with respect to polar lipid (1). The latter effect was small, but the results presented here show that it is accompanied by large changes in the fatty acid composition of the neutral lipids. Taken together the results indicate that, in the period

TABLE 1

Fatty Acid Composition of Total Neutral Lipids from Developing Herring Eggs and Early Larvae[a]

	Time after fertilization (days)										
	0	0.1	3	7	11	15	22	25	29	32	36
14:0	3.6	3.4	3.0	3.1	2.8	2.9	5.3	4.9	3.6	3.1	6.7
15:0	–	–	–	0.3	–	–	0.5	0.6	0.4	0.4	0.6
16:0	24.6	22.4	21.0	22.0	21.9	22.0	21.8	21.7	17.5	18.8	22.7
16:1(n-9)	0.7	0.8	0.9	0.8	0.8	0.9	–	1.3	0.4	0.4	–
16:1(n-7)	7.0	8.9	8.8	8.0	7.7	7.2	6.9	6.3	5.9	5.5	5.2
17:0	0.6	0.8	0.8	0.5	0.7	0.7	0.6	0.6	0.3	0.3	0.6
18:0	1.8	1.5	1.3	1.4	1.8	2.4	2.5	2.8	2.5	2.2	4.2
18:1(n-9)	18.3	30.1	31.3	27.4	24.9	22.7	18.9	17.1	15.4	14.3	11.8
18:1(n-7)	5.6	4.6	4.8	5.0	5.7	6.2	6.1	5.5	5.5	5.4	5.1
18:2(n-6)	1.5	2.9	2.6	2.2	2.1	1.8	1.3	1.2	1.1	1.0	0.9
18:3(n-3)	1.5	2.7	2.7	2.4	2.1	1.8	1.4	1.1	1.1	0.9	0.7
18:4(n-3)	1.1	1.6	1.6	1.5	1.4	1.2	1.4	1.3	1.3	1.2	0.9
20:1(n-9)	1.3	1.6	1.5	1.4	1.2	1.4	3.1	2.3	2.1	2.3	2.8
20:1(n-7)	0.1	0.6	0.6	0.5	0.8	0.8	–	–	–	–	–
20:4(n-6)	0.6	0.2	0.2	0.6	0.5	0.5	0.4	0.4	1.0	1.5	0.8
20:4(n-3)	0.6	1.0	1.1	0.9	0.9	0.8	1.0	0.9	1.0	0.9	0.8
20:5(n-3)	9.7	6.0	5.9	7.2	7.8	8.2	6.8	7.2	8.7	10.3	7.8
22:1	0.5	0.6	0.5	0.5	0.3	0.4	2.3	1.1	0.9	0.9	2.1
22:5(n-3)	0.8	0.7	0.7	0.7	0.8	0.8	0.9	0.8	1.2	1.3	0.9
22:6(n-3)	17.1	4.9	6.0	8.8	10.6	12.2	15.6	17.9	22.9	26.7	19.3
24:1	–	0.4	0.6	0.2	0.1	0.1	0.1	0.3	0.5	0.3	0.5
Total saturates	30.6	28.1	26.1	27.3	27.2	28.0	30.7	30.6	24.3	24.8	34.8
Total monoenes	33.5	47.6	49.0	43.8	41.5	39.7	37.4	33.9	30.7	29.1	27.5
Total (n-3)	30.8	16.9	18.0	21.5	23.6	25.0	27.1	29.2	36.2	41.3	30.4
Total (n-6)[b]	2.1	3.1	2.8	2.8	2.6	2.3	1.7	2.1	2.3	2.5	1.7
(n-3)/(n-6)	14.7	5.5	6.4	7.7	9.1	10.9	15.9	13.9	15.7	16.5	17.9
Total unknowns	2.8	4.5	4.2	4.7	5.3	5.0	3.0	4.2	6.5	2.3	5.6

[a]Composition as wt%. Values are means of triplicate analyses. Standard deviations are omitted for clarity but normally were <5%.

[b]Total includes 18:3(n-6) and 20:3(n-6) present in some samples at < 0.5%. Hatching occurred on day 20-21.

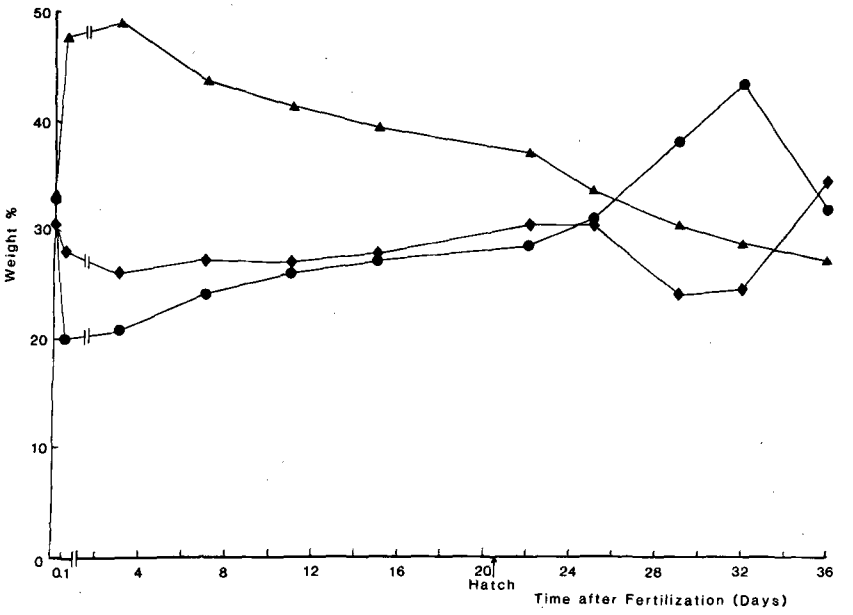

FIG. 1. Fatty acid composition of total neutral lipids from herring eggs during embryonic and subsequent early larval development. Samples of 400 eggs were taken at 0.1 and 3 days after fertilization. Samples of 200 eggs were taken at 7, 11 and 15 days. Hatching occurred on days 20-21 after fertilization, and samples of 300-500 larvae were taken on days 22, 25, 29, 32 and 36. Data are constructed from Table 1. Total polyunsaturated fatty acids had an average (n-3)/(n-6) ratio of 12.3. ● = total polyunsaturates; ◆ = total saturates, and ▲ = total monounsaturates.

immediately following fertilization, there is some utilization of neutral lipid with an apparent preferential utilization of PUFA and, to a lesser extent, saturates.

During this early period there is no significant change in the fatty acid composition of the polar lipid, which initially has a similar percentage of saturates (32%) compared with neutral lipids but a much higher percentage of PUFA (49%), a lower percentage of monoenes (15%) and an (n-3)/(n-6) ratio of approximately 30 (Table 2). The major fatty acids in the polar lipid were 16:0, 20:5(n-3) and 22:6(n-3) with significant amounts of 14:0, 16:1(n-7), 18:0 and 18:1 isomers (Table 2).

Following the initial change in the fatty acid composition of the neutral lipids, there is a progressive increase in the percentage of PUFA with a corresponding decrease in the percentage of monoenes (Fig. 1). By hatching, on days 20-21, the fatty acid composition of the neutral lipid had almost returned to that initially present in the ripe eggs (Table 1). However, the process of increasing percentage of PUFA in neutral lipid continues beyond hatching and through the early larval stages up to day 32 after fertilization, by which time PUFA have reached a much higher level and monoenes a lower level than in the original, unfertilized eggs (Fig. 1). These changes are brought about primarily by a reversal of the initial changes in the percentages of 18:1(n-9) and 22:6(n-3). However, whereas 16:1(n-7) follows this pattern, 18:1(n-7) increases during embryonic development and only decreases during larval development. The 20:2, 22:1 and 24:1 isomers, and the other major PUFA, 20:5(n-3), fluctuate without a steady pattern emerging (Table 1). Furthermore, the C_{18} PUFA do not follow the general trend and their percentage decreases (Table 1). A gradual increase in the percentage of saturates also occurs during development until yolk sac absorption, after which it declines before rising steeply on day 36 (Fig. 1). This rise in the percentage of saturates coincides with a final decrease in the percentage of 22:6(n-3), and PUFA in general, to levels similar to those in unfertilized eggs (Table 1, Fig. 1).

The changes in fatty acid composition of the neutral lipids can be correlated with the variation in the lipid class composition during this period. Polar lipid, specifically PC, is selectively catabolyzed during the period of development from 0.1 day to 35 days post-fertilization (1). The fatty acid composition data here suggest that the (n-3) PUFA produced by the hydrolysis of

TABLE 2

Fatty Acid Composition of Total Polar Lipids from Developing Herring Eggs and Early Larvae[a]

	Time after fertilization (days)										
	0	0.1	3	7	11	15	22	25	29	32	36
14:0	1.8	1.4	1.4	1.3	1.2	1.1	1.6	1.3	1.1	1.0	1.1
16:0	27.6	26.7	26.4	25.3	25.8	25.8	25.5	25.4	23.4	23.1	25.5
16:1(n-9)	0.5	0.4	0.4	0.4	0.4	0.4	–	–	0.2	0.3	–
16:1(n-7)	2.9	2.9	2.9	2.6	2.7	2.6	3.0	3.0	2.9	2.9	2.8
17:0	0.3	0.2	0.2	0.8	0.8	0.4	0.2	0.2	0.3	0.3	0.3
18:0	2.7	2.5	2.6	2.6	2.7	2.9	3.7	3.6	3.7	3.7	4.6
18:1(n-9)	4.8	4.7	4.9	4.8	5.4	5.6	6.3	6.4	7.0	7.2	7.3
18:1(n-7)	5.8	5.9	5.8	5.6	5.6	5.0	4.8	4.4	4.3	4.5	4.7
18:2(n-6)	0.5	0.5	0.5	0.5	0.6	0.6	0.6	0.7	0.7	0.7	0.7
18:3(n-3)	0.3	0.3	0.3	0.3	0.3	0.4	0.4	0.5	0.4	0.4	0.3
18:4(n-3)	0.4	0.4	0.4	0.4	0.4	0.4	0.3	0.3	0.3	0.2	0.2
20:1(n-9)	0.5	0.4	0.4	0.5	0.6	1.0	0.7	0.5	0.4	0.3	0.3
20:1(n-7)	–	0.4	0.4	0.5	0.6	0.6	–	–	–	–	–
20:4(n-6)	1.0	1.0	1.2	0.8	0.9	0.9	1.0	1.1	1.2	1.3	1.4
20:4(n-3)	0.3	0.4	0.3	0.3	0.6	0.3	0.6	0.6	0.6	0.5	0.4
20:5 (n-3)	13.7	14.1	14.3	13.7	14.3	13.7	13.9	14.3	14.1	13.2	11.6
22:5(n-3)	1.0	1.0	1.0	1.0	1.1	1.1	1.3	1.3	1.2	1.3	1.2
22:6(n-3)	31.4	30.5	32.8	32.5	32.7	32.7	32.5	34.4	35.6	37.2	35.5
24:1	0.3	0.2	0.2	0.3	–	0.3	0.5	0.5	0.4	0.2	0.3
Total saturates[b]	32.4	30.8	30.6	30.2	30.5	30.2	31.3	30.8	28.8	28.3	31.5
Total monoenes[c]	15.0	14.9	15.0	14.9	15.3	15.5	15.8	14.8	15.2	15.4	15.4
Total (n-3)[c]	47.1	46.7	49.3	48.2	49.4	48.6	49.0	51.4	52.2	52.8	49.2
Total (n-6)[c]	1.6	1.5	1.7	1.4	1.5	1.5	1.6	1.8	2.0	2.0	2.1
(n-3)/(n-6)	29.4	31.1	29.0	34.4	32.9	32.4	30.6	28.6	26.1	26.4	23.4
Total unknowns	3.9	4.6	3.7	4.6	3.3	3.7	2.3	1.2	1.8	1.5	1.8

[a]As in Table 1.

[b]Total includes 15:0 present in some samples at $< 0.5\%$.

[c]Totals include 20:1(n-11), 18:3(n-6), 20:2(n-6), 22:1 and 22:4 isomers present in some samples at $< 0.5\%$. Hatching occurred on day 20-21.

PC are not entirely oxidized but are selectively retained to some extent in the neutral lipids in exchange for monoenes mobilized from neutral lipid. In this way the higher (n-3) PUFA content of the lipids of eggs and larvae is conserved and the fatty acid composition of the neutral lipid tends to approach that of the polar lipids (Table 2). An expression of this is the (n-3)/(n-6) ratio in neutral lipid which, after its early decrease, returns to the level found in ripe eggs (Table 1).

The fatty acid composition of the polar lipids does not show a significant change throughout embryonic development, although there is a small transient increase in total PUFA content during early larval development. However, a slightly increased percentage of (n-6) PUFA generates slightly lower (n-3)/(n-6) ratios (Table 2). PC is the major phospholipid class in herring egg lipid and so the fatty acid composition of total polar lipid reflects the fatty acid composition of PC (2). The results presented here are, therefore, consistent with hydrolysis of PC to release fatty acids in the same proportion as total polar lipid, with selectivity in fatty acid utilization occurring at the stages of interconversion in the neutral lipid pool and ultimate fatty acid oxidation.

The decline in the percentage of PC had ceased between days 22 and 29 (1) although the increase in the percentage of PUFA in the neutral lipid continued during this period (Fig. 1). This is not at variance with the compositions above since selectivity in the oxidation of monoenes, as previously postulated, still may occur without a net decrease in the level of PC. Consistent with this, we noted in the preceding paper that total lipid falls during this period of starvation without greatly altering the relative amounts of total polar and total neutral lipids, i.e. there is a general reduction in all lipid classes (1).

The fatty acid composition of yolk sac polar lipids was similar to that of the larval bodies and whole larvae, with only slightly lower PUFA and higher saturates (Table 3). Little change was detected between day 1 and day 4 post-hatch. Analysis of the lipid classes in yolk sacs and larval bodies had indicated that there was movement of intact phospholipid (PC and PE) from the yolk sacs to the larval bodies (1) and

TABLE 3

Fatty Acid Composition of Polar and Neutral Lipids from Separated Herring Larvae Bodies and Yolk Sacs at 1 Day and 4 Days Post-Hatch[a]

	Polar lipids				Neutral lipids			
	Time after hatching (days)				Time after hatching (days)			
	1		4		1		4	
	Larval bodies	Yolk sacs	Larval bodies	Yolk sacs	Larval bodies	Yolk sacs	Larval bodies	Yolk sacs
14:0	1.3	2.0	1.2	1.7	4.3	7.2	4.4	7.8
15:0	0.3	0.4	0.3	0.3	0.4	0.6	0.5	1.2
16:0	23.8	28.0	24.1	25.8	19.5	21.7	20.1	20.6
16:1(n-9)	–	–	–	–	–	–	0.4	5.4
16:1(n-7)	3.2	2.5	3.1	2.6	6.3	8.1	6.4	6.5
17:0	0.3	–	0.3	0.2	0.5	0.7	0.4	1.4
18:0	3.9	3.5	3.6	4.1	2.7	2.3	2.8	3.3
18:1(n-9)	7.1	5.1	6.7	5.7	16.2	24.1	15.7	25.1
18:1(n-7)	4.4	5.4	4.3	5.1	7.2	4.7	5.9	4.1
18:2(n-6)	0.8	0.3	0.8	0.5	1.1	1.5	1.0	2.0
18:3(n-3)	0.5	0.3	0.5	0.3	1.2	1.6	1.1	1.2
18:4(n-3)	0.3	0.3	0.3	0.3	1.4	1.4	1.3	1.2
20:1(n-9)	0.3	1.4	0.4	0.9	1.9	5.2	2.3	2.3
20:4(n-6)	1.0	1.0	1.1	1.1	0.6	–	0.5	–
20:4(n-3)	0.6	0.5	0.6	0.5	0.9	1.1	0.9	0.9
20:5(n-3)	14.7	12.7	14.7	13.5	8.6	4.2	8.2	3.5
22:1	–	1.2	–	–	0.7	5.1	1.0	1.4
22:5(n-3)	1.5	1.2	1.3	1.4	1.1	0.6	1.0	–
22:6(n-3)	33.2	31.4	35.1	33.2	22.0	6.3	21.1	5.1
24:1	0.2	0.8	0.4	1.0	0.1	–	0.4	–
Total saturates	29.6	33.9	29.5	32.1	27.4	32.5	28.2	34.3
Total monoenes	15.0	16.4	14.9	15.3	32.4	47.2	32.1	44.8
Total (n-3)	50.8	46.4	52.5	49.2	35.2	15.2	33.6	11.9
Total (n-6)[b]	1.8	1.3	1.9	1.6	1.7	1.5	2.1	2.0
(n-3)/(n-6)	28.2	35.7	27.6	30.8	20.7	10.1	16.0	6.0
Total unknown	2.8	2.0	1.2	1.8	3.3	3.6	4.0	7.0

[a]As in Table 1.

[b]Totals include 18:3(n-6) and 20:3(n-6) present in some samples at < 0.5%.

the fatty acid analysis is consistent with this. Furthermore the yolk sac neutral lipids one day after hatching had low levels of 22:6(n-3), and (n-3) PUFA in general, and increased levels of saturates and monoenes, as compared with larval bodies (Table 3). The percentage of (n-3) PUFA decreased further in yolk sac neutral lipids by 4 days post-hatch. The data suggest that during embryogenesis, there is a selective transfer of neutral lipid rich in PUFA from the yolk to the larval body and that the neutral lipid remaining in the yolk sac is chiefly an energy store for the larvae, utilized after the yolk sac is absorbed and prior to the commencement of feeding. The lipid class analysis was consistent with this in that the neutral lipid in the yolk sac after hatching was conserved relative to yolk sac polar lipids and also relative to larval body neutral lipids (1).

Overall the results indicate that the high level of PUFA, laid down during vitellogenesis and present in the ripe egg at spawning, are of major importance in the embryonic and early larval development of the Atlantic herring. The PUFA are largely conserved during these developmental periods, despite specific utilization of PUFA-rich polar lipid (PC) (1) by a selective concentration of PUFA in the neutral lipid pool at the expense of monoenes. A special role for PUFA, at or immediately following fertilization, is also suggested by their sudden decrease in neutral lipid witin 0.1 day (3 hr). Finally, in an earlier study of the lipids of larval herring from hatching to 90 days post-hatch we noted an increased level of PUFA, especially 22:6(n-3) and 20:5(n-3), occurring in TAG in the first 20 days post-hatch (11). We interpreted that increase as consistent with a marked dietary input of PUFA, possibly from phytoplankton (11). The present results show clearly that the trend of increasing % of polyunsaturates in neutral lipid during the first 20 days post-hatch is in

reality a continuation of a trend established immediately post egg fertilization. As such it is unlikely to be related solely or even partly to dietary inputs in the period immediately post-hatching. The precise role of the quality and quantity of polyunsaturates in the natural diets of marine fish larvae, particularly in relation to the role these lipids play in larval survival during the critical period when the animals start to feed, remains to be elucidated.

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Inhibition of Acyl CoA: Cholesterol Acyltransferase and Sterologenesis in Rat Liver by Diazepam, *In Vitro*

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ABSTRACT

Diazepam, a commonly prescribed tranquilizer, was found to inhibit cholesterol biosynthesis in rat liver minces; inhibition appeared to occur at multiple post-mevalonate sites. Diazepam also inhibited cholesterol esterification by acylCoA:cholesterol acyltransferase in isolated liver microsomes and minces. Liver minces incubated with [^{14}C]oleate demonstrated increased uptake of the fatty acid and a greater incorporation of the substrate into triglycerides, diglycerides and phospholipids when diazepam was present. The results suggest possible mechanisms for the hypocholesterolemic effect of diazepam in experimental animals and for the elevation of triglycerides and very low-density lipoproteins in man and the rat.
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INTRODUCTION

Benzodiazepine drugs have been reported to alter the pattern of circulating plasma lipoproteins in man (1) and rat (2) and to possess antiatherosclerotic effects in rabbits (2-4) and roosters (5,6). Diazepam (the active ingredient in Valium®), the most widely used member of the benzodiazepine group, also has been reported to exert a hypocholesterolemic effect in the normal rat (2,7) and in hyperlipemic rats (8), roosters (5) and rabbits (4).

Interest in this laboratory has been on investigating the mechanisms of these diazepam effects. Our initial studies showed that diazepam inhibits plasma LCAT (lecithin:cholesterol acyltransferase, EC 2.3.1.43) in a variety of species including man (9,10) and that it also inhibits the esterification of cholesterol by arterial ACAT (acylCoA:cholesterol acyltransferase, EC 2.3.1.26) (10). In the present report, attention has been directed toward the effects of diazepam on hepatic lipid metabolism, in vitro, using rat liver minces and isolated microsomes while employing [^{14}C]mevalonate, [^{14}C]oleate and [^{14}C]oleoylCoA as substrates. The results indicate that diazepam inhibits hepatic sterologenesis at multiple sites, inhibits hepatic ACAT, and stimulates phospholipid and triglyceride synthesis. The possible relationship of these observations to in vivo effects of diazepam reported previously (1-8) is discussed.

MATERIALS AND METHODS

Animals and Tissues

Male Sprague-Dawley rats of the Upjohn strain (Upj:TUC(SD)spf, 225-250 g) were maintained on Purina Chow ad libitum. The rats were decapitated between 9 and 10 a.m. and the livers were quickly excised. The livers were rinsed in chilled 0.9% NaCl and used to prepare tissue minces (11) or homogenized in 0.1 M phosphate buffer, pH 7.4, and the microsomal fraction isolated by differential centrifugation (12).

Assay Procedure for Microsomal AcylCoA: Cholesterol Acyltransferase

Cholesterol esterification by microsomal acylCoA: cholesterol acyltransferase (ACAT) was assayed as previously described (13,14). Briefly, ACAT was assayed in a total volume of 345 μl of 0.1 M phosphate buffer (pH 7.4) containing 0.8-1.0 mg microsomal protein (15) and 0.2 μCi [1-^{14}C]oleoylCoA (sp. act. 50.0 Ci/mol, New England Nuclear Corp., Boston, Massachusetts). Diazepam (supplied by Roche Laboratories, Nutley, New Jersey) was added dissolved in 25 μl dimethylsulfoxide. Controls received 25 μl dimethylsulfoxide alone in order to internally correct for any possible solvent effects among the assays. Assays were performed in 13 × 125 mm glass tubes at 37 C for 5 min and were initiated by adding the [^{14}C]oleoylCoA in 10 μl sodium acetate buffer. The assays were terminated by the addition of 1 ml CH_3OH. The samples then were reduced in volume to about 0.2 ml under N_2 and extracted with 4 ml $CHCl_3/CH_3OH$ (2:1, v/v) according to Folch et al. (16). The lipid extracts were fractionated by thin-layer chromatography (TLC) as previously described (17) after adding unlabeled cholesteryl oleate as a carrier. The cholesteryl ester fraction was scraped from the chromatoplates (17) and assayed for radioactivity by liquid scintillation counting (18). Recovery of labeled cholesteryl

oleate was 97-98% based upon recovery of cholesteryl-[^{14}C]oleate standards.

Liver Mince Incubations

Hepatic lipid synthesis was studied in paired liver minces (500 mg) prepared from tissue taken from the central region of the large lobe (18,19). The tissue minces were incubated for periods of up to 90 min at 37 C in 3.5 ml Krebs-Ringer-bicarbonate buffer (pH 7.4) (18) containing either 2 μCi[1-^{14}C]oleic acid, sodium salt (sp. act. 52.6 Ci/mol) or 2 μCi DL-[2-^{14}C]mevalonic acid, DBED salt (sp. act. 50.1 Ci/mol) which were obtained from New England Nuclear Corp., Boston, Massachusetts. Diazepam, when present, was added to the incubations dissolved in 50 μl acetone; control incubations received 50 μl acetone alone. After incubation, tissues incubated with [^{14}C]-mevalonate were treated with alcoholic KOH to hydrolyze the tissue and to saponify the lipids as detailed previously (19). The non-saponifiable lipids were extracted with n-hexane and fractionated by TLC into C_{27}-sterols, C_{30}-sterols and squalene (19,20) and their radioactivity assayed by liquid scintillation counting as above. A portion of the non-saponifiable lipid also was redissolved in CH_3COCH_3: C_2H_5OH (1:1, v/v), treated with digitonin to precipitate cholesterol, and the cholesterol digitonides were isolated (21) and their radioactivity assayed (22). Tissues incubated with [^{14}C]oleate were extracted with $CHCl_3$/CH_3OH (2:1, v/v) (16), and the resulting lipid extract was fractionated by TLC to separate the polar and neutral lipids (23) which were scraped from the chromatoplates and analyzed for radioactivity (23).

Statistical analysis of the data was performed using Student's t-test for paired variates.

RESULTS

The addition of diazepam to isolated rat liver microsomes inhibited the esterification of cholesterol to [^{14}C]oleoylCoA by acylCoA: cholesterol acyltransferase (ACAT) (Fig. 1). The inhibition by diazepam was concentration-dependent with 50% inhibition occurring at about 0.25 mM; activity was essentially abolished at 1.0 mM. ACAT activity in microsomes re-isolated after a 5-min pre-incubation with diazepam at 37 C was reduced to a similar extent, i.e., inhibition was 42% and 70% when pre-incubation concentrations were 0.25 and 0.5 mM, respectively (mean of 2 experiements). This inhibitory activity was confirmed in liver mince preparations using [^{14}C]oleate as a substrate (Table 1). The incorporation of [^{14}C]-

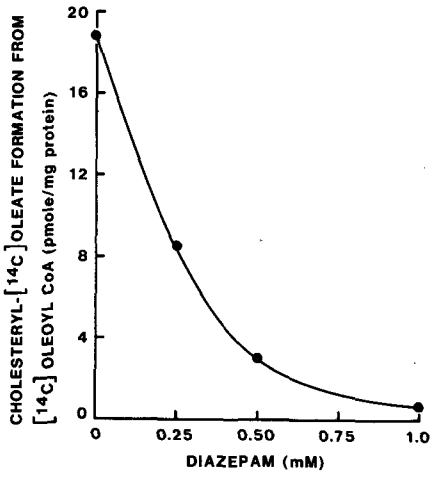

FIG. 1. Inhibition of acylCoA:cholesterol acyltransferase in isolated rat liver microsomes by diazepam. The enzyme was assayed by measuring the incorporation of [1-^{14}C]oleoylCoA into cholesteryl esters over a 5-min period at 37 C. The incubation mixtures consisted of 345 μl 0.1 M phosphate buffer (pH 7.4) containing 0.8-1.0 mg microsomal protein and 0.2 μCi [1-^{14}C]oleoylCoA (sp. act. 50.0 Ci/mol). Diazepam was added in 25 μl dimethylsulfoxide; control incubations received 25 μl dimethylsulfoxide alone. Data points are the mean of 2 experiments performed with microsomes isolated from 2 normal rat livers.

oleate into cholesteryl-[^{14}C]oleate by the liver was reduced about 64% ($P < 0.001$) by 1.0 mM diazepam in paired tissue samples. The inhibition cannot be attributed to a generalized poisoning effect since [^{14}C]oleate incorporation into the liver phospholipids and triglycerides was enhanced about 47% ($P < 0.001$).

In addition to inhibiting cholesterol esterification, diazepam was found to be an inhibitor of sterologenesis as well. A time-course for the incorporation of [^{14}C]mevalonate into squalene, C_{30}-sterols, C_{27}-sterols and digitonin-precipitable sterols in paired liver minces in the presence of 0.5 mM diazepam is shown in Figure 2. Inhibition of [^{14}C]mevalonate incorporation into the cholesterol precursors squalene and the C_{30}-sterols was evident within 15 min after diazepam. By 30 min, a 32% inhibition of [^{14}C]mevalonate incorporation into the C_{27}-sterol fraction of the diazepam-treated tissue became apparent as well. Between 15 and 90 min, incorporation into the C_{27}-sterol fraction of the diazepam-treated tissue rose a mere 50% from 4,500 to 6,800 dpm/g, while incorporation into the same fraction in control tissue increased about 5-fold, from 4,600 to 23,500 dpm/g. In tissue samples incubated for 90 min, the inhibition of incorpo-

TABLE 1

Effect of Diazepam on the Incorporation of [^{14}C]oleate into Lipids of Rat Liver Minces in vitro (dpm/mg wet weight)

	Phospholipid	Diglyceride	Triglyceride	Free fatty acid	Cholesteryl ester
Control	191[a] ±9	51 ±3	529 ±36	687 ±50	22 ±2
Diazepam (1.0 mM)	281[b] ±8	100[b] ±4	777[b] ±44	1004[b] ±39	8[b] ±1

Liver minces (500 mg) prepared from paired tissue samples from normal male rats were incubated for 90 min at 37 C in 3.5 ml Krebs-Ringer-bicarbonate buffer (pH 7.4) containing 2 μCi [1-^{14}C]oleic acid sodium salt (sp: act. 52.6 Ci/mol) in the presence and absence (control) of 1.0 mM diazepam.

[a]Values are means ± SEM of 7 animals/group.

[b]Differs significantly ($P < 0.001$) from control values using Student's t-test for paired variates.

ration into C_{27}-sterols and squalene was about 70% and 66% respectively in the presence of diazepam. A reduction in the formation of digitonin-precipitable sterol in diazepam-treated tissue at all time points further confirmed inhibition of sterologenesis. In control tissues, approximately 80% of the radioactivity of the C_{27}-sterol fraction was precipitable as cholesterol digitonide, whereas in the treated tissues only about 20% was digitonin precipitable. These data specifically indicate inhibition of cholesterolgenesis by diazepam.

DISCUSSION

The studies presented here identify several alterations to hepatic lipid metabolism that occur in the presence of diazepam. Most notable, perhaps, is the inhibitory effect of diazepam on cholesterol esterification and on cholesterol biosynthesis. From the data presented in Figure 2, it appears that the overall effect of diazepam on sterologenesis results from inhibition at multiple sites in the sterol biosynthetic pathway. The first site lies between mevalonate and squalene, as evidenced by inhibition of labeled squalene formation from labeled mevalonate. A second site appears to lie between lanosterol and the C_{27}-sterols. Evidence for the second site comes from a calculation of the ratio of labeled C_{30}-sterol: C_{27}-sterol in control vs. diazepam-treated tissue pairs at each time interval. In control tissues, the ratio declined stepwise from 1.8 at 15 min to 0.5 at 90 min, whereas in the treated tissue the ratio increased from 1.1 to 1.8 over the same period, indicating a disproportionate accumulation of radioactivity in C_{30}-sterols vs C_{27}-sterols in the treated tissue. The similarity

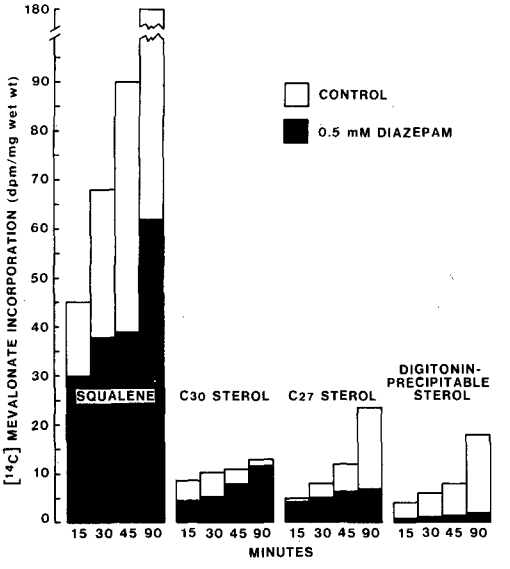

FIG. 2. Inhibition of sterologenesis from [2-^{14}C]-mevalonic acid in rat liver minces by diazepam. Minces (500 mg) from paired samples of liver were incubated at 37 C for periods of 15-90 min with (■) and without (□) 0.5 mM diazepam in 3.5 ml Krebs-Ringer-bicarbonate buffer (pH 7.4) that contained 2 μCi [2-^{14}C]-mevalonic acid, DBED salt (sp. act. 50.1 Ci/mol). Data for each time period are means of 2 experiments.

in C_{30}-sterol labeling at 90 min in the control and diazepam-treated tissue (12,800 vs 11,900 dpm/g, respectively) is probably a reflection of the cumulative decrease in flow-through of C_{30}-sterol to C_{27}-sterol with diazepam treatment as opposed to some other effect, such as a reversal of the inhibition of C_{30}-sterol formation seen at 15, 30, and 45 min. The inhibition of cholesterol synthesis observed may offer an

explanation for the plasma cholesterol lowering effect of orally administered diazepam in normal (2,7) and in Triton W1339-induced hyperlipidemic rats (8). Inhibition of sterologenesis, however, is unlikely to be important as a mechanism of cholesterol-lowering observed with diazepam treatment in cholesterol-fed roosters (5) and rabbits (4). Conceivably, cholesterol lowering under those conditions could be a result of decreased absorption of cholesterol through inhibition of intestinal ACAT (24,25). Data presently available on the effects of diazepam in man suggest that the drug neither inhibits sterologenesis, as determined with liver biopsy material (26), nor affects plasma cholesterol levels (26). These differences in drug effect between man and rat could reflect inherent differences in the enzymes involved or factors such as differences in drug distribution and metabolism.

The dual effects of cholesterol synthesis inhibition and ACAT inhibition shown by diazepam in liver preparations have been observed previously with other membranophilic agents such as lidocaine (19) and chlorpromazine (18,23). The blockade of sterol synthesis by these agents also occurs at multiple sites in the biosynthetic pathway beyond HMGCoA reductase (3-hydroxy-3-methylglutaryl coenzyme A reductase, EC 1.1.1.34) (18,19). The fact that these agents have little structural similarity suggests that their effects on the enzymes may be indirect rather than direct. Since ACAT and the enzymes of the post-mevalonate stages of sterologenesis reside in the endoplasmic reticulum, the possibility that the drugs exert their effects by affecting the physical state of the membrane (e.g., change lipid fluidity or bilayer thickness) seems plausible (19,27); the fact that ACAT inhibition persisted after re-isolation of diazepam-treated microsomes is not inconsistent with this view. There is evidence which suggests that alterations in membrane lipid fluidity can affect the conformation of particulate enzymes and thus alter their rates of catalyses (28-30). The fact that ACAT and various particulate enzymes of the sterol synthesis pathway are affected similarly by the 3 drugs also could indicate a close topological proximity of the enzymes in the membrane or that the enzymes have similarly structured lipid boundary layers which are affected in a similar fashion by the drugs (31,32).

Other effects of diazepam on liver lipid metabolism presented here include the stimulation of [^{14}C]oleate incorporation into the glycerolipids (phospholipids, diglycerides and triglycerides). While the precise mechanism of these effects is not known, it seems probable that the increased incorporation into the glycerolipids could be accounted for on the basis of increased availability of [^{14}C]oleate substrate, i.e., diazepam-treated tissues accumulated significantly higher levels ($P < 0.001$) of unesterified [^{14}C]oleate during incubation but the percentage distribution of [^{14}C]oleate incorporated into those fractions was similar to controls.

Plasma VLDL (very low density lipoprotein), whose major lipid component is triglyceride, has been reported to increase in patients receiving benzodiazepine therapy (1). Although the mechanism of this response is unknown, it has been suggested that it could reflect increased triglyceride synthesis secondary to increased plasma-free fatty acid levels arising from drug-stimulated lipolysis (1). The stimulation of hepatic triglyceride synthesis from fatty acid observed in the present studies also suggests a mechanism for the hypertriglyceridemic effect of diazepam in normal rats (8). In addition to a direct stimulation of hepatic triglyceride synthesis, diazepam may indirectly promote VLDL-triglyceride elevations by impairing VLDL clearance. Evidence for this possibility comes from observations that diazepam is an inhibitor of LCAT (lecithin: cholesterol acyltransferase, EC 2.3.1.43) in plasma from man and rat (9,10); LCAT, in conjunction with lipoprotein lipase, participates in the normal catabolism of VLDL (33).

Because the present studies are short-term in design, correlations of the observations with effects observed in the long-term in man and animals (1-8) must be viewed cautiously. Despite this limitation, the data presented suggest a mechanistic basis of cause-effect relationships which may be explored more fully with different experimental approaches.

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Action of Cobra Venom Phospholipase A_2 on Large Unilamellar Vesicles: Comparison with Small Unilamellar Vesicles and Multibilayers

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ABSTRACT

Phospholipase A_2 *(Naja naja naja)* catalyzes the hydrolysis of dipalmitoyl phosphatidylcholine in small unilamellar vesicles (SUVs) with a faster initial rate than in large unilamellar vesicles (LUVs) and multilamellar vesicles (MLVs). For the SUVs, the hydrolysis was initially faster for gel phase than liquid crystalline phase phospholipid. For both LUVs and MLVs, hydrolysis was low except in a small temperature range around the thermotropic phase transition of the phospholipid. In this temperature range, the reaction time course of phospholipase action on dipalmitoyl phosphatidylcholine in LUVs and MLVs included a lag period. With SUVs, a lag period also was observed above the phase transition temperature, but it was not observed below it.
Lipids 20:80-83, 1985.

INTRODUCTION

Phospholipase A_2 (EC 3.1.1.4) from *Naja naja naja* cobra venom is a small, water-soluble enzyme that catalyzes the hydrolysis of the *sn*-2 fatty acid ester of phosphatidylcholine (PC) to form lysophosphatidylcholine (LPC) and fatty acid. It preferentially catalyzes the hydrolysis of gel phase dimyristoylphosphatidylcholine (DMPC) or dipalmitoylphosphatidylcholine (DPPC) in small unilamellar vesicles (SUVs) under initial rate conditions, with the activity immediately above the phase transition temperature (T_c) being 1/2 to 1/3 of the activity immediately below the transition (1). The activity at T_c is not enhanced over that expected from the temperature dependence observed below T_c. However, *Naja naja naja* phospholipase A_2 catalyzes the hydrolysis of 1,2-dimyristoyl-*sn*-glycero-3-phosphorylcholine (DMPC) in multibilayers or multilamellar vesicles (MLVs) very poorly, except in a small temperature range around T_c (1). This enhanced activity near T_c is similar to the results observed for the action of the phospholipases A_2 from pancreas, bee venom and *Crotalus atrox* venom on various DMPC and 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine (DPPC) vesicle preparations after long time periods of reaction (2-4).

The activity of the cobra venom enzyme evidently is very sensitive to the aggregation state and phase of PC with saturated fatty acid chains. The differing activity of this enzyme toward PC in SUVs and MLVs could result from packing differences in the highly curved outer surface of the SUVs (5) compared to the relatively flat, planar outer surface of the MLVs, but other factors such as the larger number of layers, slower rate of diffusion and greater heterogeneity in sizes of the MLVs could be responsible. It was therefore of interest to determine the dependence of the phospholipase A_2 activity on the phase of PC in large unilamellar vesicles (LUVs) which could more directly be compared with the highly curved SUVs. We report here the reaction time courses of the phospholipase A_2 catalyzed hydrolysis of DPPC in LUVs at temperatures above, below and at the phase transition and compare them with those of SUVs and MLVs.

EXPERIMENTAL PROCEDURE

MLVs of L-α-DPPC (Calbiochem) were prepared by suspending the dry phospholipid in buffer (100 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$) by repeated pipetting of the solution while it was maintained above T_c. SUVs were prepared by sonication of the MLVs above T_c, centrifugation and annealing as described previously (1). LUVs were prepared by the following modification of the alcohol injection method (6). DPPC in absolute ethanol (75 mM PC) was allowed to flow by gravity through disposable 1-ml syringes attached to luer-lock syringe needles (28 gauge, Hamilton N-72822) which were inserted approximately 1 cm into a rapidly stirred buffer solution (100 ml) maintained ato 50 C. The average flow rate of each syringe was 10 μl/min; the combined flow rate from several syringes was under 50 μl/min. The final volume of ethanol did not exceed 7.5% of

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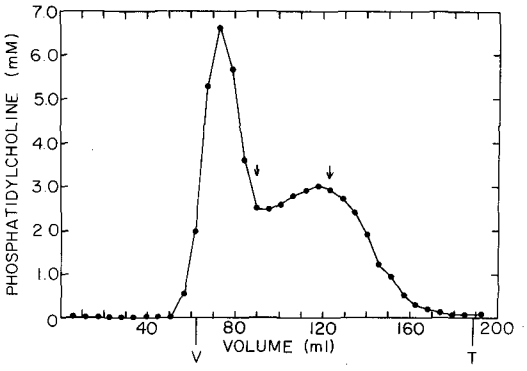

FIG. 1. Elution profile of DPPC LUVs (303 μmol applied in 10 ml) on CL-Sepharose 2B (2.0 cm × 50 cm). The temperature was maintained at 50 C. V and T refer to void and total volumes, respectively. The vesicles used for the phospholipase A_2 assays were taken from the fractions bounded by and including those marked by the arrows.

the volume of the vesicle solution. The vesicles were dialyzed extensively to remove the ethanol and concentrated in an Amicon ultrafiltration apparatus with an Amicon XM-100 or Nucleopore type C (molecular weight cutoff 100,000) ultrafiltration membrane. The vesicles were then chromatographed on a CL-Sepharose 2B gel filtration column in order to isolate a relatively homogeneous population of LUVs. The buffer consisted of 100 mM Tris-HCl, pH 8.0, and 10 mM $CaCl_2$. The first portion of the peak eluting in the internal volume was concentrated by ultrafiltration (2-3 fold) and used immediately for the enzyme assay.

Phospholipase A_2 was purified from *Naja naja naja* cobra venom as described by Deems and Dennis (7) and Darke et al. (8). Assays were conducted by adding the phospholipase in 10 μl of water to 1.00 ml of the appropriate vesicle preparation in buffer (100 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$) at the temperature indicated. The final DPPC concentration in each preparation was determined by a phosphate analysis (9). The hydrolysis of PC was followed by phosphate analysis of the reaction products, PC and LPC, after they were extracted into organic solvent and separated by thin-layer chromatography (TLC) (1).

RESULTS AND DISCUSSION

Figure 1 shows a typical elution profile on Sepharose CL-2B of large unilamellar vesicles of DPPC prepared by the ethanol injection method. The fraction bounded by the arrows was used for the phospholipase A_2 assays. This sample contained vesicles ranging in size from 540 Å to 1260 Å in diameter with an average diameter of 760 Å (determined by negative staining electron microscopy). Thus, the LUVs used here were larger in size than the highly curved sonicated vesicles (250 Å diameter (10)), but were much smaller and more homogeneous than multibilayer preparations (5,000 − 20,000 Å diameter (11)).

The time courses for phospholipase A_2 action on this fraction of LUVs and on SUVs of DPPC are shown in Figure 2. The initial activity was highest toward DPPC in SUVs at all temperatures tested. However, the reaction time course toward DPPC in LUVs was biphasic at temperatures near T_c (which is about 42 for DPPC in MLVs (12)) with an acceleration of hydrolysis such that the PC in LUVs was hydrolyzed faster than that in SUVs at long reaction times. At lower temperatures such as 36 and 21, the reaction did not appear biphasic even after 121 min of hydrolysis at the latter temperature, although an eventual acceleration could not be ruled out. For both the small and large unilamellar vesicles, a stimulation of activity after a lag period was observed above T_c. However, this stimulation was not observed with the small vesicles at low temperatures, using either the thin-layer chromatography assay at fixed time points as indicated in the present study or the continuous pH-stat assay for the first minutes of reaction (1). The reaction time courses on DPPC LUVs were very similar to those observed on DPPC MLVs which also exhibit biphasic reaction time courses near T_c (Fig. 3). The peak of hydrolysis of DPPC in both LUVs and MLVs after long reaction times was highest at temperatures around the phase transition. From the similarity of the reaction time courses, it is apparent that the action of *Naja naja naja* phospholipase A_2 is affected by the physical state of DPPC in LUVs and MLVs in a similar fashion.

It should be noted that in the initial stages of the reaction, the vesicles are presumed to remain intact. Thus, only the outer monolayer of phospholipid (68% for SUVs, 55% for LUVs of 760 Å diameter, and 10 to 15% for MLVs) is exposed to the enzyme. The amount of phospholipid used in each experiment was set so that the concentration of exposed phospholipid (that on the outside of the vesicle) was roughly equal at the start of each kinetic experiment. During the course of the reaction, the fraction of PC hydrolyzed eventually exceeded the fraction of phospholipid on the outer monolayer, suggesting either an increased permeability to the phospholipase A_2 or a rearrangement of the vesicle structure to allow hydrolysis of the inner layer or layers.

With all vesicles, the initial enzyme activity

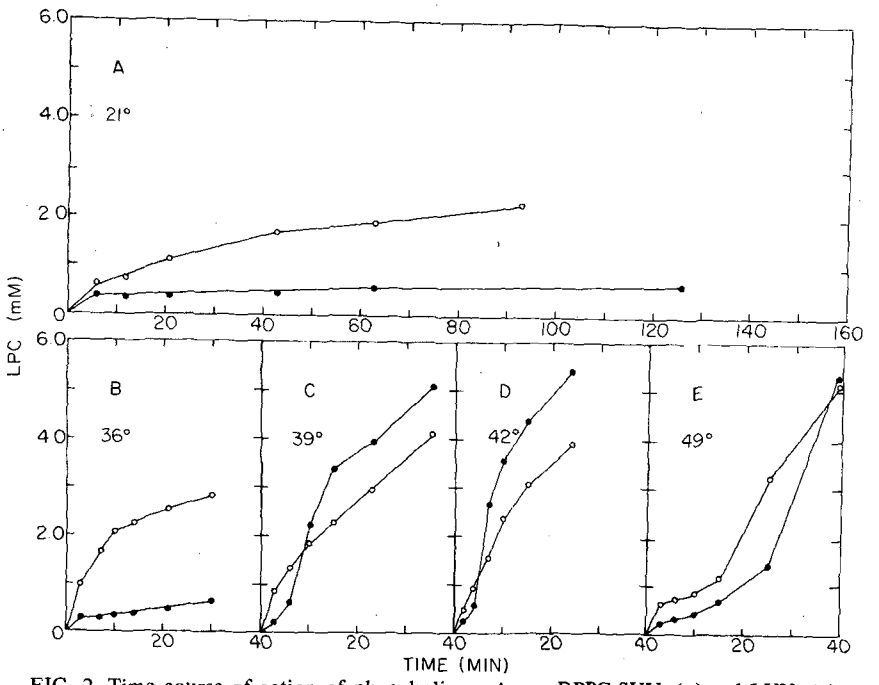

FIG. 2. Time course of action of phospholipase A_2 on DPPC SUVs (○) and LUVs (●) at various temperatures: (A) 21 C, (B) 36 C, (C) 39 C, (D) 42 C, and (E) 49 C. The production of LPC is shown. The concentration of DPPC in the SUV preparation was 5.5 mM and in the LUV preparation was 6.9 mM. The concentration of enzyme employed was 0.82 μg/ml.

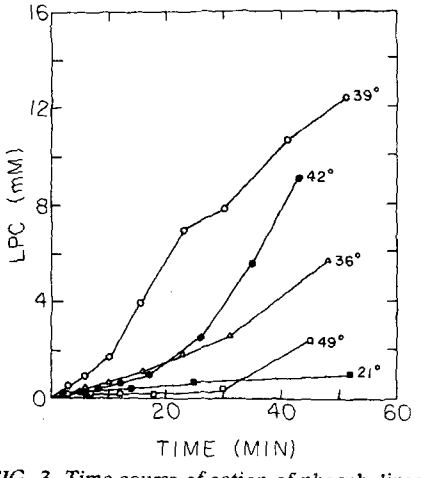

FIG. 3. Time course of action of phospholipase A_2 toward DPPC MLVs at 5 temperatures: 21 C (■), 36 C (△), 39 C (○), 42 C (●), and 49 C (□). The production of LPC is shown. In the assays, the concentration of DPPC was 23.9 mM and the concentration of enzyme was 0.82 μg/ml.

appears to be optimal at temperatures in which the packing of PC is imperfect. For example, gel phase SUVs have been postulated to contain circular planar regions or "facets" of about 75 Å in diameter in a polyhedral arrangement with packing irregularities or defects in the regions which join the adjacent facets (13). The PC molecules in these edge regions may contain a higher fraction of gauche bonds which can pack into these regions. Experimental support for this is provided by Raman spectroscopy, which showed a higher fraction of gauche bonds for gel phase SUVs compared to gel phase MLVs (14). Below the pretransition temperature, the PC in MLVs is packed tightly with the chains tilted; between the pretransition and T_c, the bilayers become rippled (15). Similar packing is expected for PC in LUVs, as the phase transition behavior is similar to that of MLVs (16). A pretransition also is observed in SUVs (17). In MLVs, LUVs and SUVs, there is a coexistence of the gel and liquid crystalline phases at T_c itself. These regions also may be imperfectly packed (as with the planar regions of the gel phase vesicles). Thus, there is defective packing in the gel phase of SUVs and at T_c itself. These regions also may be imperfectly packed (as with the planar regions of the gel phase vesicles). Thus, there is defective packing in the gel phase of SUVs and at T_c for MLVs and LUVs as well as SUVs. It has been suggested that the pancreatic phospholipase A_2 favors gel phase SUVs as a substrate over liquid crystalline phase SUVs because of the defects in the packing of the gel phase vesicles (18). This enzyme may prefer the phase transition

temperature of other vesicle preparations because of the coexistence of 2 phases, due to an increased penetration of the enzyme into the bilayer (2). While this explanation also may account for the results reported here for the *Naja naja naja* phospholipase A_2, it also is possible that the rate of hydrolysis of gel phase PC in SUVs is higher than in the liquid crystalline phase because of contributions from other subtle differences in phospholipid conformation or the properties of the phospholipid/water interface such as surface polarity, hydration, or [H+] concentration. These are important in the non-enzymatic hydrolysis of phospholipids (19) as well as in the action of phospholipase A_2 on micellar interfaces (20).

An apparent increase in activity after long time periods of hydrolysis is observed above T_c for DPPC dispersed in small and large unilamellar vesicles and multibilayers. There are several explanations for this. It could be the end of an initial slow step resulting in an apparent lag phase, such as the pre-steady state suggested for the action of pancreatic phospholipase A_2 on PC monolayers (21), which for SUVs may be rate-limiting above the phase transition, but not below it (18). However, it also may be due to an effect of the reaction products in the bilayer by either their direct interaction with the enzyme or by their alteration of the physical state of the remaining phospholipid substrate due to changes in phospholipid phase or aggregation (3). Fatty acids are known to raise the phase transition temperature of saturated phosphatidylcholine (22). As fatty acid and LPC accumulate in the bilayer, the PC eventually may go into the gel phase or be at the transition at the higher temperature due to an increase in T_c. This would cause a stimulation of the activity if the enzyme prefers small unilamellar vesicles in the gel phase and multibilayers at the transition temperature (3). Yet another possibility is that a phase separation of one or both of the products into a region of the vesicle distinct from the PC region occurs. The phase separation would result in the co-existence of 2 regions (similar to the situation at the phase transition) with defective packing between the regions. If phospholipase A_2 activity is indeed correlated with the presence of defective packing, a phase separation would be expected to increase the phospholipase A_2 activity.

The present study has shown that the reaction time courses of the phospholipase A_2 toward bilayer PC is quite different for gel phase SUVs and gel phase LUVs, where there is a difference in the packing of the outer surface of phospholipid. The activity clearly is highest on phases which contain defective packing, but further work is required to confirm whether or not this preference of the enzyme is for all PC phases with defective packing and to determine whether this preference is related to binding of the enzyme to the surface, phospholipid, or some other factors similar to those occurring with micellar interfaces (20).

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Lipid Class Composition during Embryonic and Early Larval Development in Atlantic Herring (*Clupea harengus*, L.)

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ABSTRACT

The lipid class compositions of Atlantic herring eggs and larvae were determined immediately before fertilization, after fertilization and at various times during subsequent embryonic and early larval development. Total lipid constituted 15% of the dry wt of ripe eggs, 70% of the total lipid being polar lipid with phosphatidylcholine (PC) accounting for almost 90% of the polar lipid. In general, the total lipid content decreased gradually during embryogenesis and in particular during larval development. Within 3 hr after fertilization the relative percentage of neutral lipid decreased slightly. This was followed by a general decrease in polar lipid which, by the stage of yolk sac absorption, was reduced to 52% of the total lipid. The decreased percentage of polar lipid was due entirely to a decrease in PC, which was reduced to 66% of the polar lipids at the stage of yolk sac absorption. The accompanying increase in the percentage of neutral lipids was mainly due to increased percentages of triacylglycerols (TAG) up to yolk sac absorption and cholesterol esters in the larval stages. During the first 4 days after hatching, phospholipids and to a lesser extent cholesterol were preferentially depleted in the yolk sacs, which also had higher levels of free fatty acids. The results are discussed in relation to possible roles of different lipids during embryonic and early larval development.
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INTRODUCTION

The lipids in many marine teleost eggs are rich in phospholipids (1-4). In a recent survey we found that in teleost eggs with relatively short incubation periods (up to 20 days), e.g. cod *(Gadus morhua)*, haddock *(Melanogrammus aeglefinus)*, whiting *(Merlangus merlangus)*, saithe *(Pollachius virens)*, and herring, phospholipid accounted for 62-72% of the total lipid (4). PC was the major phospholipid class in each case, ranging from 63-83% of the total phospholipid (4). However, marine teleost eggs with relatively longer incubation periods (over 20 days), e.g. sand eel *(Ammodytes lancea)* and capelin *(Mallotus villosus)*, had higher lipid levels, with neutral lipids, mainly TAG, accounting for up to 77% of the total lipid (4). These findings suggest that in marine teleost eggs with relatively short incubation periods the majority of the lipid may be destined for biomembrane formation rather than energy production during development.

Studies of energy metabolism in developing fish eggs have been concerned mainly with measuring levels of potential energy reserves, metabolites and relevant metabolic enzyme systems (5-8). Deuchar (5) concluded that the order of utilization of energy reserves in teleost fish eggs was carbohydrate, then protein and finally lipid. This was questioned by Terner (6) on the basis of testing the ability of various externally added substrates to stimulate respiration in developing trout eggs. Acetate, a product of fatty acid catabolism, stimulated respiration to a greater extent than glycolytic substrates, including glucose (9). Radioactive acetate also was incorporated into polar lipid and subsequently into neutral lipid (10). Furthermore, the glycogenolytic and gluconeogenic pathways, although apparently operative in developing trout eggs, could not account for all of the endogenous respiration (11). More recently Vetter et al. (12) studied energy metabolism including lipid utilization in the rapidly developing egg of a marine teleost, the red drum *(Sciaenops ocellata)*, and established that lipid was utilized from both polar and neutral fractions throughout the developmental period.

The present study was undertaken to determine changes in the lipid class composition during embryonic and early larval development in a typical marine teleost, Atlantic herring, which has a high content of phospholipid (70% of total lipid) in its egg. The aim was to elucidate possible different roles of the various lipid classes during this period.

MATERIALS AND METHODS

Eggs. Roe was excised from ripe Atlantic herring *(Clupea harengus)* caught at the end of March 1983 on the Ballantrae Bank in the lower Clyde Estuary, Scotland. A batch of

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unfertilized eggs was retained and the remainder fertilized with milt taken from 5 ripe males. The fertilized eggs were allowed to attach to glass plates and were maintained in outside tanks supplied with running sea water at an ambient temperature of approximately 8-10 C. Samples of 200-400 eggs were taken at time intervals during embryonic development, frozen at -15 C for 1-2 weeks and finally stored at -70 C for 6 mo prior to analyses.

Larvae. Roe was collected and eggs fertilized and maintained as above during March and April 1984. Hatching occurred after 20-21 days, and approximately 300-500 larvae were collected at time intervals and immediately frozen and stored in liquid nitrogen before transfer to the -70 C freezer. Yolk sacs were separated from the bodies of further samples of approximately 250-300 larvae before freezing in liquid nitrogen. Liquid nitrogen treatment was necessary with larvae due to the presence of highly active phospholipases which may maintain residual activity at -15 C. The sea water supplied to the tanks was passed through a 64 μm filter which removed all zooplankton, although phytoplankton passed through.

Analytical. Moisture contents were determined after freeze drying samples in an Edwards "Speedivac" centrifugal freeze drier. Total lipid was extracted by the method of Folch et al. (13), determined gravimetrically and stored in chloroform/methanol (2:1, v/v) containing 0.05% butylated hydroxytoluene at -20 C between analytical procedures.

Lipid Analyses. Lipid class analyses were carried out using Chromarods-SII and an Iatroscan TH-10 Mark II analyzer (Iatron Laboratories Inc., Tokyo) coupled to a Hewlett Packard 3390A recording integrator. Samples of 0.5 μl containing approximately 50 μg of total lipid were spotted on the origins of the rods using disposable 0.5 μl micropipettes (Camlab Ltd., Cambridge, U.K.). To analyze polar lipid classes the rods were developed in chloroform/methanol/distilled water (70:35:3.5, v/v/v). To analyze neutral lipid classes the rods were developed in hexane/diethyl ether/formic acid (85:15:0.04, v/v/v). The former procedure separated individual phospholipid classes together with neutral lipids as a single unresolved zone. The latter procedure separated individual neutral lipid classes together with phospholipids as a single unresolved zone. Developed rods were dried at 100 C for 2 min before being scanned at 3.1 mm sec^{-1}. The flame ionization detector was operated at a hydrogen pressure of 0.71 kg cm^{-1} and an air flow of 2000 ml min^{-1}. The rods were stored in a constant humidity chamber between analyses and were scanned twice before each development and analysis. Peak areas for each lipid class obtained from the integrator were converted into μg of lipid using calibration curves constructed from standard solutions of known concentrations and with compositions similar to those of the experimental samples analyzed. Data obtained from scanning 18 individual rods were used for each single analytical determination.

RESULTS AND DISCUSSION

Upon release and fertilization there is an increase in the water content of the eggs (Table 1), a phenomenon noted in the past (14). The presumed uptake of water is rapid in the initial phase of development and essentially complete by 3 days. Thereafter the water content remains relatively constant up to yolk sac absorption after day 25. The initial uptake of water could reflect simply a difference in osmolarity between the sea water and the eggs on spawning (15) or be a residual trace of the hydration that occurs, during maturation of marine teleost eggs, including demersal eggs (16,17). During the first 3 days the lipid content as a per cent of the egg dry weight shows a downward trend, although the lipid content increases again at 7 days (Table 1). Thereafter the per cent total lipid in the developing eggs fluctuates, although the value immediately after hatching increases 2-fold, due to the loss of much of the dry weight in the form of the chorion and associated material. The chorion alone can account for up to 1/3 of the dry wt of unfertilized herring eggs and, presumably, an even greater proportion after 3 weeks of development (18). Throughout early larval development the lipid content falls if no food is available to the larvae, as is the case here (Table 1). Overall, and despite some fluctuations, the results indicate that lipid is utilized during embryonic development and this utilization is accelerated in the early larval stages. This is consistent with the results of Boulekbache, who detected increasing lipid metabolism as embryonic development continued (7).

The results for changes in the proportion of total polar and total neutral lipids as well as the individual lipid classes can be considered, for convenience, in 4 developmental periods. The first of these is 0.1 day (approximately 3 hr) after fertilization of the eggs. The percentage of neutral lipids decreases and the percentage of polar lipids increases during this period (Table 1). The change is small but in this single sample set is statistically significant ($p<0.001$, Student t test, $t_{(28)} = 7.01$). The change in the relative proportions of polar and neutral lipids is re-

TABLE 1

Lipid Content and Lipid Class Analysis from Developing Herring Eggs and Larvae[a]

	Time after fertilization (days)										
	0	0.1	3	7	11	15	22	25	29	32	36
Moisture content (%)[b]	74.3	82.1	87.7	87.7	85.8	87.8	85.3	87.3	91.1	91.2	89.8
Lipid content (% dry wt)[b]	14.6	12.8	9.4	12.3	10.7	11.4	23.7	22.0	19.4	14.2	10.0
% Polar lipids	68.9 ± 1.4	71.6 ± 0.5	68.3 ± 0.7	64.9 ± 0.9	63.6 ± 0.5	57.6 ± 0.8	54.9 ± 2.7	52.5 ± 1.8	57.8 ± 1.5	57.0 ± 2.5	52.0 ± 1.3
% Neutral lipids	31.1 ± 1.4	28.4 ± 0.5	31.7 ± 0.7	35.1 ± 0.9	36.4 ± 0.5	42.4 ± 0.8	45.1 ± 2.7	47.5 ± 1.8	42.2 ± 1.5	43.0 ± 2.5	48.0 ± 1.3
Neutral classes (% total lipid)											
Triacylglycerol	13.7 ± 0.3	12.8 ± 0.9	15.1 ± 0.5	17.0 ± 0.9	17.8 ± 0.7	20.1 ± 1.3	22.9 ± 1.6	25.0 ± 0.9	22.0 ± 0.7	17.4 ± 1.1	12.9 ± 0.8
Cholesterol/DAG	8.6 ± 0.2	8.2 ± 0.6	9.1 ± 0.3	9.3 ± 0.3	9.6 ± 0.5	10.8 ± 0.8	8.9 ± 1.1	11.2 ± 0.7	13.7 ± 0.6	14.2 ± 0.6	18.2 ± 1.3
Free fatty acids	6.2 ± 0.3	4.7 ± 0.5	5.2 ± 0.3	6.1 ± 0.4	6.4 ± 0.7	8.4 ± 0.8	5.7 ± 0.6	4.5 ± 0.7	1.9 ± 0.4	3.1 ± 0.7	9.3 ± 0.7
Sterol esters	2.7 ± 0.2	2.6 ± 0.1	2.3 ± 0.2	2.6 ± 0.5	2.6 ± 0.2	3.1 ± 0.3	7.2 ± 1.2	5.8 ± 0.4	4.5 ± 0.3	7.9 ± 1.1	7.2 ± 0.5
Polar classes (% total lipid)											
Phosphatidylcholine (PC)	61.9 ± 2.0	62.4 ± 0.5	60.4 ± 0.9	58.0 ± 0.8	55.8 ± 0.7	48.7 ± 1.0	40.1 ± 2.7	41.0 ± 2.0	43.4 ± 1.2	37.6 ± 1.8	34.3 ± 1.1
Phosphatidylethanolamine	3.8 ± 0.5	6.0 ± 0.2	4.7 ± 0.4	4.0 ± 0.3	4.9 ± 0.3	5.3 ± 0.5	8.7 ± 1.0	9.5 ± 1.5	12.2 ± 0.9	16.4 ± 1.7	12.0 ± 1.0
Phosphatidylserine	0.7 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.8 ± 0.2	1.0 ± 0.2	1.4 ± 0.5	1.1 ± 0.7	1.5 ± 0.6	2.1 ± 1.0	4.3 ± 1.2
Phosphatidylinositol	0.5 ± 0.3	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.2	0.4 ± 0.2	1.0 ± 0.5				
Sphingomyelin	0.8 ± 0.3	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.2	1.1 ± 0.2	1.5 ± 0.6	0.7 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	1.4 ± 0.7
Lyso - PC	1.4 ± 0.4	1.3 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	0.9 ± 0.2	1.0 ± 0.2	2.2 ± 0.6	0.2 ± 0.1	N.D.	0.2 ± 0.1	N.D.

[a]Values are means ± standard deviations from 15-18 determinations unless stated otherwise.

[b]Results from one determination.

N.D. = Not detected. Traces of fatty alcohol < 0.5% were found in most samples.

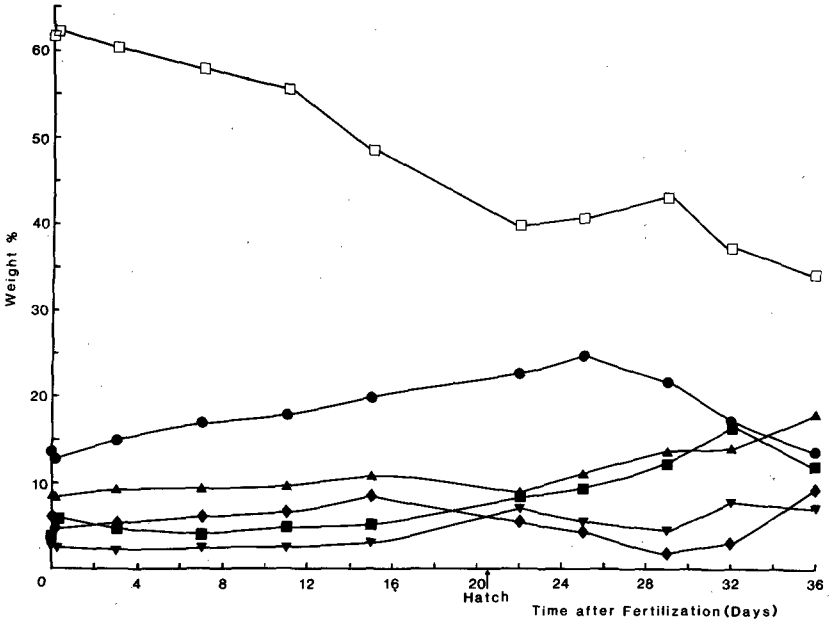

FIG. 1. Major lipid class composition of herring eggs during embryonic and subsequent early larval development. Samples of eggs and larvae were taken at the times indicated as described in Materials and Methods. □ = phosphatidylcholine; ■ = phophatidylethanolamine; ● = triacylglycerol; ▲ = cholesterol/diacylglycerol; ◆ = free fatty acids, and ▼ = sterol esters.

flected in the percentages of the individual neutral and major phospholipid classes (Table 1, Fig. 1).

The second developmental period is between 0.1 day and 15 days when there is a progressive decrease in the percentage of polar lipid, due entirely to a decreased percentage of PC, accompanied by a reciprocal increase in total neutral lipids, predominantly TAG (Table 1, Fig. 1). During the third developmental period, from day 15 to day 29, the proportion of polar lipids initially continues to decline but then increases due to a slight reversal in the decrease of PC and an increase in the percentage of phosphatidylethanolamine (PE) (Table 1, Fig. 1). Similarly, the increase in the percentage of TAG continues initially and then is reversed after yolk sac absorption, which had occurred in over 95% of larvae sampled on day 29 after fertilization. Hatching occurs in the middle of this period without any major changes in the lipid class composition other than the trends already noted and a 2-fold rise in sterol esters (Table 1, Fig. 1).

During the final developmental period, after yolk sac absorption, the percentage of total polar lipids decreases again due to a renewed decrease in PC and also PE in the later stage (Table 1, Fig. 1). TAG decreases sharply throughout this period and free fatty acids (FFA) increase some 4-fold from day 29 to day 36. Diacylglycerol (DAG) could not be resolved consistently from cholesterol during the analytical procedure used with the Iatroscan in the present study. However, examination of the total lipid by TLC-densitometry revealed an increase in DAG during this period, probably underlying the increased percentage of cholesterol/DAG recorded in Table 1 and Figure 1. Combined with the decreasing lipid content of the larvae (Table 1), these results suggest a general mobilization and utilization of both polar and, increasingly, neutral lipids in these starving larvae.

The lipids of the yolk sac in newly hatched (day 1) larvae show a class composition very similar to the larval bodies (Table 2). However, there are relatively high levels of lyso-PC and FFAs in the yolk sac lipids, probably reflecting the pattern of lipid mobilization at that time. At this stage the yolk sacs are large and can account for up to 50% of the dry wt of the larvae (19). By 4 days post-hatch (day 25 after fertilization) the yolk sacs are considerably depleted of polar lipids, in particular PC and PE, but of the neutral lipid classes, only cholesterol/DAG decreases (Table 2). This, combined with corresponding increases of these components in the larval bodies (Table 2) and the increase of PC, PE and cholesterol/DAG in the whole larvae (Fig. 1) during this period, suggests movement of intact biomembrane components from the yolk sac to the larval body. By this time the

TABLE 2

Lipid Class Analyses of Separated Larval Bodies and Yolk Sacs at 1 Day and 4 Days Post-Hatch[a]

	Time after hatching (days)			
	1		4	
	Larval bodies	Yolk sacs	Larval bodies	Yolk sacs
% Polar lipids	53.1 ± 2.6	57.5 ± 2.8	55.0 ± 1.8	37.9 ± 1.7
% Neutral lipids	46.9 ± 2.6	42.5 ± 2.8	45.0 ± 1.8	62.1 ± 1.7
Neutral classes (% total lipids)				
Triacylglycerol	25.6 ± 1.6	18.7 ± 1.6	23.7 ± 0.6	33.3 ± 2.4
Cholesterol/DAG	10.9 ± 0.7	6.1 ± 1.7	12.5 ± 0.4	4.3 ± 0.7
Free fatty acids	2.7 ± 0.8	10.2 ± 0.4	3.2 ± 0.5	12.3 ± 1.8
Sterol esters	7.0 ± 1.4	7.5 ± 1.0	5.2 ± 0.4	9.7 ± 1.0
Fatty alcohols	0.7 ± 0.4	N.D.	0.4 ± 0.2	2.5 ± 1.0
Polar classes (% total lipid)				
Phosphatidylcholine (PC)	40.1 ± 3.0	40.1 ± 2.3	42.3 ± 2.1	33.5 ± 1.1
Phosphatidylethanolamine	9.4 ± 0.9	7.8 ± 1.3	10.8 ± 1.7	1.9 ± 0.8
Phosphatidylserine	1.5 ± 0.6	] 2.3 ± 0.8	] 1.2 ± 0.9	0.3 ± 0.1
Phosphatidylinositol	1.0 ± 0.4			0.4 ± 0.1
Sphingomyelin	1.3 ± 0.6	1.8 ± 0.5	0.7 ± 0.2	0.7 ± 0.3
Lyso - PC	N.D.	5.5 ± 1.4	N.D.	1.3 ± 0.5

[a]As in Table 1.

N.D. = Not detected.

yolk sac has decreased generally to only 10-20% of the total dry wt of the larvae (19), and the total lipid content of the whole larvae has decreased also (Table 1). Therefore, significant amounts of lipid are still being catabolized, reflected in the continued presence of lyso-PC and even higher levels of FFAs in the yolk sac (Table 2).

Overall the results indicate that after fertilization there is a very early, short period where some neutral lipid is utilized, predominantly FFA and TAG. Subsequent to this there is overall, a preferential net consumption of a single phospholipid class, PC, rather than neutral lipid which is conventionally regarded as more of an energy reserve than phospholipid. The major result of this process is that PC, which in the unfertilized eggs originally constituted almost 90% of the total phospholipids, is reduced by the time of yolk sac absorption to a level where the composition of the phospholipid pool is more characteristic of that found in most biological membranes. The precise reasons underlying the preponderance of PC in the released eggs are not clear at the present time. However, in addition to providing fatty acids, utilization of PC provides inorganic phosphate for intermediary metabolism including nucleic acid synthesis as well as choline for, possibly, C1 (methyl) metabolism (20) and neurotransmission. The 2-fold increase in TAG during embryonic and early larval development suggests that this lipid is preferentially retained up until yolk sac absorption, when it becomes an important energy source for the larvae until feeding commences.

The results presented here show net consumption of the polyunsaturated fatty acid-rich (4) phospholipid PC during embryonic and early larval development in Atlantic herring. One possible consequence of this strategy could be the catabolism of essential polyunsaturated fatty acids, deposited and concentrated in the eggs, that would have been expected to be conserved for biomembrane formation. The fatty acid compositions of the polar and neutral lipid fractions throughout embryonic and early larval development in Atlantic herring are the subject of a further report (21).

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Comparative Studies of Triacylglycerol Structure of Very Low Density Lipoproteins and Chylomicrons of Normolipemic Subjects and Patients with Type II Hyperlipoproteinemia

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ABSTRACT

The triacylglycerols of very low density lipoproteins (VLDL) and of chylomicrons were analyzed in the fasting and postabsorptive states from normolipemic subjects and patients with Frederickson's Type II hyperlipoproteinemia, who subsisted on free choice diets, standard diets excluding lard, or were given a breakfast enriched in lard. The VLDL and chylomicrons were obtained by conventional ultracentrifugation, and the triacylglycerols were isolated by thin-layer chromatography (TLC). Representative sn-1,2-, sn-2-3- and sn-1,3-diacylglycerols were generated by partial Grignard degradation of the triacylglycerols and a stereospecific hydrolysis by phospholipase C of the mixed sn-1,2(2,3)-diacyl phosphatidylcholines prepared as intermediates. Representative sn-2-acylglycerols were obtained by hydrolysis with pancreatic lipase. Positional distribution of the fatty acids was established by subtracting in turn the fatty acid composition of the sn-2-position from the fatty acid composition of the sn-1,2- and sn-2,3-diacylglycerols. The molecular association of the fatty acids in the diacylglycerol moieties was determined by gas-liquid chromatography with mass spectrometry (GC/MS) of the tertiary-butyldimethylsilyl (t-BDMS) ethers. The molecular association of the fatty acids in the triacylglycerols was determined by 1-random 2-random 3-random calculation following experimental validation of the distribution. The results confirm a marked asymmetry in the positional distribution of the fatty acids in all triacylglycerol samples, with the palmitic acid predominantly in the sn-1-position, the unsaturated acids about equally divided between the sn-2- and sn-3-positions, and the stearic acid divided about equally between the sn-1- and sn-3-positions. The overall structure of the VLDL and chylomicron triacylglycerols from patients and control subjects was characterized by a non-correlative distribution of fatty acids under all dietary conditions.
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INTRODUCTION

Previous studies of the composition (1-5) and stereospecific distribution (2-4) of fatty acids in the VLDL-TG and total plasma TG have shown significant differences between patients with hyperlipoproteinemia and normolipemic subjects. Thus, Gordon et al. (3) have reported that Type II patients and normal subjects on a controlled diet differ in the asymmetry of distribution of linoleic acid in the sn-1- and sn-3-positions of the VLDL and LDL, while structural analyses of plasma total triacylglycerols by Parijs et al. (2,4) have shown statistically significant differences between normal subjects and Type IV patients for palmitic and linoleic acids in the sn-2-, and for oleic acid and linoleic acid at the sn-3-position, when subsisting on free choice diets. Recently we have determined the positional distribution of the fatty acids in the triacylglycerols and the molecular association of the fatty acids in the sn-1,2(2,3)- and sn-1,3- diacylglycerols of VLDL of normal subjects and patients with Type III and Type IV hyperlipoproteinemia (6). Although we confirmed the overall asymmetry of distribution of the fatty acids, in all triacylglycerol samples, we could not obtain any evidence for significant differences in the positional distribution or molecular association of the fatty acids between the normal subjects and patients. In all instances the triacylglycerol structures agreed closely with those calculated on the basis of the 1-random 2-random 3-random distribution. In the present study we have compared the structures of the VLDL and chylomicron triacylglycerols from normal subjects and patients with Type II hyperlipoproteinemia on free choice and controlled diets, as well as following a lard challenge. The results confirm the high asymmetry of the triacylglycerols in the VLDL and chylomicrons of both normal subjects and patients, but fail to show structural differences following correction for discrepancies in the fatty acid composition. It is concluded that patients with hyperlipoproteinemia synthesize and secrete triacylglycerols of apparently normal molecular structure. A preliminary report has appeared (7).

MATERIALS AND METHODS

Standards

Purified synthetic sn-1,2-, sn-2,3- and sn-1,3-diacylglycerols containing the common fatty acids were available from a previous study (8). Reference fatty acid methyl esters, synthetic triacylglycerols and diacylphosphatidylcholines were obtained from Supelco, Bellefonte, Pennsylvania. Phospholipase C *(Clostridium perfringens)* was from Sigma Chemical Co., St. Louis, Missouri. The chemical reagents and solvents were as described previously (6).

Subjects and Samples

The plasma samples were obtained from 4 normolipemic subjects and 4 patients (30-59 years old) with Type II hyperlipoproteinemia at St. Michael's Hospital Lipid Clinic, Toronto, Ontario. The type of hyperlipoproteinemia of each patient was established on the basis of clinical and biochemical criteria published by the Lipid Research Clinics Program (9). The subjects and patients were taken off all lipid lowering medication and therapeutic diets. They were instructed to follow a free choice diet for one month and were seen at weekly visits for fasting blood samples, weight checks and diet interviews (Visit 1). After determining each individual's energy intake, the subjects were placed on a standardized diet for 2 weeks (Visit 2). The diet consisted of 45% of energy as fat, 40% as carbohydrate and 15% as protein. The fat intake came entirely from beef, chicken, eggs, olive oil, butter and 2% milk. Hydrogenated, highly polyunsaturated fat and long chain fatty acids were avoided. The chicken, beef and olive oil were weighed and measured by the dietitian, and these foods, as well as rye and Italian bread, were given to the subjects for the 2-week period. The subjects were asked to abstain from alcohol during this period. Blood samples (20 ml) were taken after a 12-hr fast at 7, 10 and 14 days, and the subjects' compliance to diet and weights were checked at each visit. On the final day of the 2-week period, following a fasting blood sample, the subjects ate a special 1300-calorie breakfast containing 74 g of fat from a standard lard obtained from a single batch (Visit 3). A second blood sample was taken 4 hr after the breakfast. The blood samples were not pooled.

Preparation of Lipoproteins

The chylomicron, VLDL, LDL and HDL fractions were isolated essentially according to Hatch and Lees (10), as described in detail previously (11). The identity of the lipoproteins was confirmed by determination of protein and the total lipid profile. The protein concentration in the lipoprotein fractions was determined by the method of Lowry et al. (12), using bovine serum albumin as standard. The preparations of chylomicrons and the VLDL were extracted with diethyl ether after the color development. The total lipid profiles of the different lipoprotein classes were determined as described previously (11). Figure 1 gives the total lipid profiles of the VLDL and chylomicron fractions of representative control subjects and patients with Type II hyperlipoproteinemia.

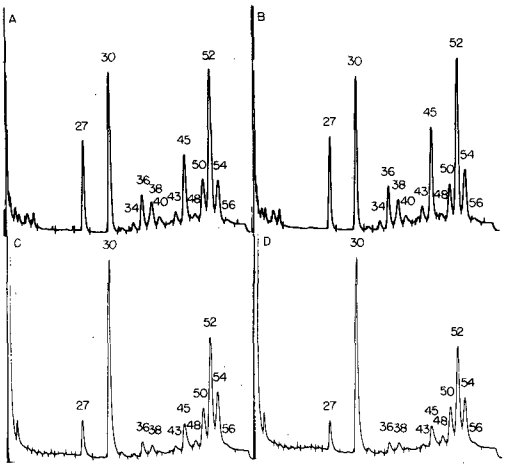

FIG. 1. Total lipid profiles of VLDL and chylomicrons from representative control subjects and patients with Type II hyperlipoproteinemia. A, VLDL of control subject; B, VLDL of Type II patient; C, Chylomicrons of control subject; D, chylomicrons of Type II patient. Peak 27, TMS ether of cholesterol; Peak 30, tridecanoylglycerol internal standard; Peak 34, TMS ether of palmitoylsphingosine; Peaks 36-40, TMS ethers of diacylglycerols of a total of 34-38 acyl carbons; Peaks 43-45, cholesteryl esters of fatty acids with a total of 16-18 acyl carbons; Peaks 48-56, triacylglycerols with a total of 48-56 acyl carbons. Sample size: 1 μl of an approximately 1% solution in silylation mixture. Attenuation: 100 times full sensitivity. Other operating conditions as given in text.

Analysis of Lipids

Total lipid extracts of the various lipoprotein fractions were obtained by a modification of the method of Folch et al. as previously described (11). The triacylglycerols were isolated by preparative TLC on silica gel H plates with petroleum ether (b.p. 30-60 C)-diethyl ether (150:20, v/v) as the developing solvent. About 5-15 mg of triacylglycerol were obtained. Stereospecific analyses of the total triacylglycerols were performed according to Myher and Kuksis (13). Mixed sn-1,2(2,3)-

diacylglycerols were generated by a partial Grignard degradation of the triacylglycerols as described by Yurkowski and Brockerhoff (14), but the proportions of the sample and reagents were scaled down for smaller quantities of material (13). The diacylglycerols resulting from the reaction were isolated by TLC using borate-impregnated silica gel G and chloroform-acetone (97:3, v/v) as the developing solvent to give pure sn-1,2(2,3)- and sn-1,3-diacylglycerols (15). The mixed sn-1,2(2,3)-diacylglycerols were converted into the sn-1,2(2,3)-diacylphosphatidylcholines using a modification of the original procedure of Baer and Kindler (16). The mixed diacylphosphatidylcholines were purified and isolated by TLC using silica gel H and chloroform-methanol-acetic acid-water (25:45:12:6, v/v/v/v) as the developing solvent (17). The sn-1,2- and sn-2,3-diacylglycerols were released from the mixed phosphatidylcholines by stepwise hydrolysis with phospholipase C as previously described (13). The released sn-1,2- and sn-2,3-diacylglycerols were purified separately by TLC on borate impregnated silica gel G and were recovered and used for the determination of the composition and molecular association of the fatty acids as described below. The fatty acid composition of the 2-monoacylglycerols was obtained independently by hydrolysis of triacylglycerols with pancreatic lipase (18), using a diethyl ether-extracted porcine pancreatic lipase, *tris*(hydroxymethyl)-aminomethane buffer and gum Arabic (Analabs, North Haven, Connecticut). The monoacylglycerols were isolated by TLC on borate impregnated silica gel G with chloroform-acetone (88:12, v/v) as the developing solvent (15). Intact triacylglycerols and diacylglycerol-t-BDMS ethers were resolved according to their acyl carbon number by GLC on 3% OV-1 (19). The molecular species of the various diacylglycerol preparations were determined by GC/MS of their t-BDMS ethers as previously described (20,21). The t-BDMS ethers were prepared by reacting the diacylglycerols with tertiary-butyldimethyl chlorosilaneimidazole reagent (Applied Science Laboratories, State College, Pennsylvania) at 80 C for 20 min (20). Before GLC, the t-BDMS ethers of diacylglycerols were purified by TLC using toluene-diethyl ether (97:3, v/v) as the developing solvent. Fatty acid methl esters were analyzed on conventional GLC columns packed with 10% EGSS-X and 3% SILAR 5CP as previously described (22). The fatty acid methyl esters were prepared using 6% H_2SO_4 in absolute methanol or 1N NaOMe in methanol-benzene (60:40, v/v) as the catalyst.

TABLE 1

Fatty Acid Composition of VLDL and Chylomicron* Triacylglycerols of Control Subjects and Patients with Type II Hyperlipoproteinemia

		Fatty acids							
Subjects	Diets	14:0	16:0	16:1	18:0	18:1	18:2	18:3/20:1	20:3/20:4
		Mole %							
Controls									
L1858	FC	2.8	26.2	4.6	2.0	22.6	29.7	1.4	0.8
L1690	FC	1.6	26.7	4.4	5.5	41.0	18.1	1.8	0.9
L1689	FC	2.0	31.3	4.7	2.8	41.4	16.1	1.2	0.5
Type II									
L1899	FC	1.0	27.7	5.6	2.2	40.4	19.9	1.0	1.0
M0009	FC	1.0	26.1	3.6	2.6	37.0	26.1	1.4	1.0
M0010	FC	1.7	28.6	4.4	2.4	37.2	21.9	1.5	1.3
Controls									
L1824	LD	1.5	32.2	3.6	5.6	42.7	12.6	1.2	0.7
L1825	LD	1.5	29.3	4.7	4.5	44.3	13.6	1.4	0.8
L1824*	LD	1.5	27.9	3.4	10.0	43.5	11.4	1.2	1.0
L1825*	LD	1.7	26.3	3.2	11.3	43.2	11.6	1.3	0.9
Type II									
L1924	LD	1.9	28.2	4.0	5.1	42.7	16.2	1.1	0.9
M0044	LD	1.1	26.2	3.2	2.5	39.8	23.4	1.2	1.3
M0046	LD	1.0	26.1	3.2	4.0	37.9	24.4	1.5	1.1

FC = free choice diets; fasting plasma.
LD = breakfast including lard; plasma 4 hr after meal.

Calculations

The fatty acid composition of the sn-1- and sn-3-positions were taken as the average values calculated by 2 methods (13). The composition of the sn-1-position was derived by subtracting the composition of position 2 from that of the sn-1,2-diacylglycerols and by subtracting the composition of the sn-2,3-diacylglycerols from the composition of the triacylglycerols. Similarly, the composition of the sn-3- position was derived by subtracting the composition of position 2 from that of the sn-2,3-diacylglycerols and by subtracting the composition of the sn-1,2-diacylglycerols from the composition of the triacylglycerols. Theoretical compositions of triacylglycerols and diacylglycerols were calculated on the basis of 1-random 2-random 3-random distribution (8), which assumes that the fatty acid composition of each position of the acylglycerol molecule is independent of the composition of the other 2 positions. The corresponding carbon number and double bond number proportions of the triacylglycerols and sn-1,2-, sn-2,3- and sn-1,3-diacylglycerols were calculated by summing and normalizing the appropriate products of multiplication. The determined fatty acid compositions of the various diacylglycerols and of the total triacylglycerols were cross-checked by appropriate algebraic methods using various combinations of the fatty acid composition of the mono-, di- and triacylglycerols as outlined previously (13,23,24). An index of nonrandomness (INR) of distribution of molecular species of diacylglycerols was computed (6) by the formula

$$INR = \Sigma (Expt. - Calc.)^2$$

where Expt. and Calc. are the experimentally determined and the calculated compositions of the molecular species, and the summation is made over the entire series of molecular species. A value close to zero indicates a complete randomness. Duplicate analyses of a complex mixture of natural diacylglycerols give INR values of 0-20. Values exceeding twice the error of determination were considered significantly different from the calculated distribution (6).

RESULTS

Analysis of Total Triacylglycerols

Table 1 gives the fatty acid composition of the triacylglycerols recovered from the VLDL fractions of 3 control subjects and patients with Type II hyperlipoproteinemia on a free choice diet and of VLDL and chylomicron fractions of two control subjects and patients after a breakfast containing large amounts of lard. The fatty acid compositions are closely similar among the triacylglycerols of both lipoprotein classes of all the subjects despite the lack of deliberate dietary control, except for control subject L1858, whose VLDL triacylglycerols were much richer in 18:2 and poorer in 18:1 than the corresponding samples from other subjects. There was also a significantly higher proportion of 18:0 in the chylomicron triacylglycerols when compared to the VLDL triacylglycerols of the same subjects. We have shown elsewhere (11) that the fatty acid composition of the VLDL triacylglycerols is closely similar to that of the LDL and any HDL triacylglycerols of the same subject. However, the fatty acid composition of the chylomicron triacylglycerols usually differs significantly from that of the VLDL triacylglycerols.

Table 2 gives the carbon number distribution of the VLDL and chylomicron triacylglycerols for the control subjects and patients with Type II hyperlipoproteinemia. All samples contain the bulk of the molecular species in the form of C_{52} triacylglycerols, which represent combinations of one C_{16} with two C_{18} acids. The C_{50} and C_{54} species are made up of two

TABLE 2

Carbon Number Distribution in Triacylglycerols of VLDL and Chylomicrons* from Normolipemic Subjects and Patients with Type II Hyperlipoproteinemia

		Carbon numbers					
Subjects	Diets	46	48	50	52	54	56
Controls							
L1689	FC	0.8	4.1	20.3	61.2	12.8	0.5
L1690	FC	0.6	4.3	18.3	54.1	20.8	1.9
L1858	FC	0.6	4.4	17.5	59.3	16.3	1.9
Type II							
L1899	FC	0.4	2.8	18.2	58.9	17.4	2.3
M0009	FC	0.2	1.9	13.7	59.8	21.7	2.7
M0010	FC	0.4	3.2	16.7	58.2	18.9	2.5
Controls							
L1824	LD	0.1	2.5	17.4	62.7	14.7	2.7
L1825	LD	0.2	2.8	17.9	59.9	17.0	2.2
L1824*	LD	0.3	3.3	18.1	50.3	25.0	3.0
L1825*	LD	0.5	3.5	18.2	48.3	26.8	2.8
Type II							
M0044	LD	0.3	2.2	14.6	60.0	20.5	2.5
M0046	LD	0.3	2.3	15.0	59.7	20.5	2.3
Controls							
L1822	CD	0.3	3.2	19.2	57.1	17.9	2.4

FC = free choice diet, fasting plasma.
LD = breakfast with lard, plasma 4 hr after meal.
CD = controlled diet, fasting plasma.

TABLE 3

Positional Distribution of Fatty Acids in VLDL and Chylomicron* Triacylglycerols of Control Subjects and Patients with Type II Hyperlipoproteinemia

Sn-position	Diet	Fatty acids										
		14:0	15:0	16:0	16:1	17:0	18:0	18:1	18:2	18:3	20:3	20:4
		Moles %										
Controls												
L1822	CD											
1		3.0	1.1	62.8	5.5	1.1	5.5	15.9	3.6	1.6		
2		0.8	0.3	12.8	4.8	0.4	2.8	60.8	14.9	1.1	0.1	1.2
3		1.0	0.2	8.9	2.9	0.1	4.2	64.3	14.5	2.9	0.6	0.6
L1824	LD											
1		2.5		69.7	3.5		8.1	12.1	2.8	0.6	0.1	0.4
2		1.6		21.2	5.0		3.3	50.2	17.3	0.9	0.01	0.5
3		0.3		5.7	2.3		5.3	65.7	17.6	2.1	0.3	0.8
L1824*	LD											
1		1.9		40.7	3.1		16.9	29.7	6.0	1.0		0.6
2		1.8		33.2	4.3		4.8	41.1	13.4	0.9		0.4
3		0.8		9.8	2.8		8.5	59.6	17.7	1.8	0.6	1.4
L1825*	LD											
1+3		1.4		19.4	2.5		14.6	47.5	11.6	1.6	0.2	1.2
2		2.3		41.6	4.7		4.6	34.4	14.7	0.7	–	–
Type II												
L1899	FC											
1		2.0	0.6	63.5	6.9	0.5	3.6	15.5	6.4	1.0	–	–
2		1.0	0.2	12.4	6.4	0.2	1.6	51.2	24.9	1.0	0.1	1.1
3		0.4	0.1	8.3	3.6	0.03	2.1	55.4	26.0	2.2	0.4	1.3
M0009	FC											
1		2.0	0.7	63.2	5.2	0.7	4.8	15.1	7.1	1.3	–	–
2		0.4	0.1	7.7	3.6	0.1	1.2	44.4	39.7	1.4	0.2	1.3
3		0.6	0.1	7.8	1.9	0.2	2.6	51.6	31.3	2.5	0.6	1.1
M0010	FC											
1		3.2	0.7	67.0	5.5	0.7	3.8	12.7	5.5	1.1	–	–
2		0.9	0.3	8.3	5.2	0.1	1.8	49.3	30.8	1.6	0.3	1.3
3		0.9		10.7	2.6	0.1	2.8	51.5	26.5	2.8	0.5	1.6
M0044	LD											
1		2.0	0.5	62.7	4.1	0.6	5.0	16.6	6.0	1.5	0.1	0.9
2		1.1	0.4	12.2	4.1	0.1	1.5	44.4	34.5	0.8	0.3	0.8
3		0.4	0.3	3.6	1.3	0.2	2.0	58.0	29.5	2.7	0.3	1.7
M0046	LD											
1		1.9	0.3	57.7	4.0	0.6	7.6	17.4	8.2	1.5	0.1	0.7
2		0.8	0.6	16.1	3.2	0.2	1.9	42.8	33.4	0.5	0.1	0.5
3		0.4	0.1	4.3	2.4	0.2	3.4	53.2	31.5	2.8	0.5	1.4

FC, CD and LD as explained in Table 2.

C_{16} and one C_{18} acids, and of three C_{18} acids, respectively, and also contribute significant proportions of mass to the total triacylglycerols. Smaller amounts of species of C_{46} and C_{48} triacylglycerols also are seen. These represent mainly combinations of one C_{14} with two C_{16}, and three C_{16} acids, respectively. The VLDL triacylglycerols possess similar carbon number profiles for all subjects, which must be due to the consumption of more or less similar diets by these subjects. The consumption of a single meal of lard brings about little change in the overall distribution of the carbon numbers of the VLDL triacylglycerols. The carbon number distribution of the chylomicron triacylglycerols, however, has been altered markedly, resulting in a significant reduction in the proportion of the C_{52} triacylglycerols and an increase in the C_{54} triacylglycerols, when compared with the corresponding VLDL triacylglycerols. These changes apparently reflect the presence of increased amounts of dietary stearic acid as already noted in Table 1.

Table 3 gives the positional distribution of

TABLE 4

Relative Distribution of Fatty Acids in the sn-1, sn-2- and sn-3-positions of VLDL and Chylomicron* Triacylglycerols from Normal Subjects and Patients with Type II Hyperlipoproteinemia

Subjects/ Patients	Diet	Sn-position 1-	2-	3-	Sn-position 1-	2-	3-
				Moles %			
		18:1			18:2		
Controls							
L1822	CD	11.3	43.1	45.6	11.0	45.2	43.7
L1824	LD	9.5	39.2	51.3	7.5	45.8	46.6
L1824*	LD	22.8	31.5	45.7	17.6	39.3	43.1
Type II							
M0009	FC	13.6	40.0	46.5	9.1	50.8	40.1
M0010	FC	11.2	43.4	45.4	8.7	49.1	42.2
L1899	FC	12.7	41.9	45.4	11.1	43.5	45.4
M0044	LD	14.0	37.3	48.8	8.6	49.2	42.1
M0046	LD	15.4	37.7	46.9	11.1	45.7	43.2
		16:0			16:1		
Controls							
L1822	CD	74.3	15.2	10.5	41.4	36.0	21.8
L1824	LD	72.1	22.0	5.9	32.4	46.5	21.1
L1824*	LD	48.6	39.6	11.7	30.7	42.3	27.0
Type II							
M0009	FC	80.3	9.8	9.9	46.7	33.5	17.8
M0010	FC	77.9	9.6	12.4	41.2	39.2	19.7
L1899	FC	75.4	14.8	9.8	40.6	38.1	21.3
M0044	LD	79.8	15.6	4.6	43.3	43.4	13.3
M0046	LD	73.9	20.6	5.5	42.3	33.1	24.6
		18:0			18:3		
Controls							
L1822	CD	46.7	18.8	34.4	32.1	16.0	52.1
L1824	LD	48.8	19.6	31.5			
L1824*	LD	55.9	16.0	22.1			
Types II							
M0009	FC	58.5	10.9	30.6	26.1	26.9	47.0
M0010	FC	47.1	18.0	34.8	27.2	26.3	46.7
M1899	FC	49.1	18.4	32.2	23.1	27.9	48.7
M0044	LD	62.1	12.7	25.2	23.1	10.4	66.4
M0046	LD	61.3	12.6	26.1	11.0	8.8	80.2
		14:0			18:3/20:1		
Controls							
L1822	CD	63.1	16.2	20.7	32.1	15.9	52.0
Type II							
M0009	FC	66.0	14.7	19.3	26.1	26.9	47.0
M0010	FC	63.6	18.6	17.8	27.2	26.2	46.6
L1899	FC	58.9	28.6	12.5	23.1	28.0	48.9
M0044	LD	62.1	24.8	13.1	32.2	18.8	49.0
M0046	LD	58.0	30.6	11.4	35.0	10.6	54.4

Abbreviations as explained in Table 2.

fatty acids in the VLDL and chylomicron triacylglycerols of the control subjects and patients with Type II disease. The distribution of the fatty acids is highly asymmetrical in all VLDL and chylomicron samples, which confirms the results of previous analyses (3). The sn-1-position is occupied largely by palmitic acid, while the sn-2- and sn-3-positions are preferentially esterified with oleic and linoleic acids in about equal proportions. The amount

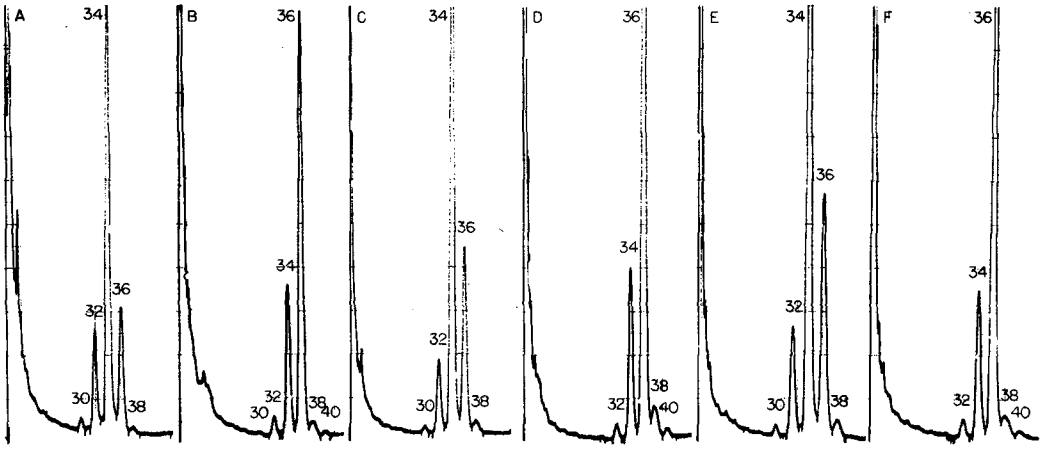

FIG. 2. Carbon number profiles of sn-1-2- and sn-2,3-diacylglycerol moieties of VLDL triacylglycerols of a control subject and a patient with Type II hyperlipoproteinemia. A, sn-1,2-diacylglycerols of control subject; B, sn-2,3-diacylglycerols of control subject; C, sn-1,2-diacylglycerols of Type II patient; D, sn-2,3-diacylglycerols of Type II patient; E, sn-1,2-diacylglycerols of the same patient after a lard meal; F, sn-2,3-diacylglycerols of the same patient after a lard meal. Peaks 30-40, TMS ethers of diacylglycerols with a total number of 28-38 acyl carbons. GLC conditions as given in text. Meals and diacylglycerol preparations as described in text.

of stearic acid is only slightly greater in the sn-1 position than in the sn-3 position of the glycerol molecule. There are no major consistent differences found in the positional distribution of the fatty acids in the VLDL triacylglycerols between control subjects and Type II patients.

Table 4 shows the per cent distribution of the major and some minor fatty acids in the 3 positions of sn-glycerol. Although the diet affects the total content and quantitative positional distribution of a given fatty acid, it has much less influence upon the overall structure of the triacylglycerol molecules, with the relative distributions of the fatty acids among the 3 positions of the glycerol molecule remaining little affected. A similar distribution is seen for the fatty acids in the chylomicron samples, except for an elevation of palmitic acid in position-2. Gordon et al. (3) had reported previously that the VLDL-TG of control subjects and of Type II patients contained about 5% of the 18:2 in sn-1-position and 40% in sn-3-position, which is of the order observed here.

Analyses of Derived Diacylglycerols

Figure 2 compares the carbon number distribution in the sn-1,2- and sn-2,3-diacylglycerol moieties of the triacylglycerols of VLDL and chylomicrons from representative subjects. The marked differences in the patterns reflect the overall asymmetry of the fatty acid distribution in the triacylglycerols. Table 5 gives the calculated and determined distribution of the sn-1,2-diacylglycerols derived from the VLDL triacylglycerols of the control subjects and patients with Type II disease along with the theoretical composition obtained by the 1-random 2-random 3-random distribution. There is a reasonably close agreement between the experimental and the calculated composition of the sn-1,2-diacylglycerols of both control subjects and patients, although the non-randomness indices (INR) in 2 instances exceed significantly the values observed for replicate analyses of the same sample. Likewise, there were significant differences between the calculated and determined compositions of the sn-1,2-diacylglycerol moieties of the VLDL and chylomicron triacylglycerols (results not shown). Apparently, the fatty acids are esterified to each position of the glycerol molecule on the basis of their relative masses without regard for the composition of the other positions. It should be noted, however, that in case of the chylomicrons, the non-correlative distribution refers to the random reesterification of the sn-2-monoacylglycerols or of the sn-2,3-diacylglycerols with the fatty acids in the sn-1-position (23), while in the VLDL of hepatic origin, the non-correlative distribution would refer to the independent acylation of the sn-1- and sn-2-positions of the glycerol phosphate.

Table 6 shows the calculated and determined composition of the molecular species of the sn-2,3-diacylglycerol moieties of the corresponding triacylglycerols. Again, the indices of non-randomness in 2 instances exceed several times the values observed for duplicate analyses of the same sample. In most instances, however, the indices are within the range of duplicate

TABLE 5

Molecular Species of sn-1,2-diacylglycerols Derived from VLDL Triacylglycerols of Normal Subjects and Patients with Type II Hyperlipoproteinemia

Molecular species	L1822*		L1899**		M0009**		M0010**		M0044***		M0046***	
	Exptl.	Calc.	Exptl.	Calc.	Exptl.	Calc.	Exptl.	Calc.	Exptl.	Calc.	Exptl.	Calc.
						Mole %						
30:0	1.2	0.9	0.8	0.9	0.5	0.4	1.0	0.9	0.8	0.9	0.7	0.7
30:1	0.3	0.2	0.6	0.2	0.3	0.1	0.5	0.2	0.3	0.1	0.3	0.1
32:0	7.8	8.2	7.3	8.0	4.2	4.9	5.3	5.8	5.8	7.7	5.6	9.4
32:1	6.9	5.7	7.1	6.1	5.2	3.6	6.0	5.6	4.4	4.2	4.3	3.4
32:2	0.9	0.7	0.8	1.0	0.7	1.0	1.7	1.3	1.2	0.9	1.2	0.8
34:0	2.7	1.9	–	1.2	1.8	0.9	0.3	1.2	1.8	1.2	3.5	2.1
34:1	46.1	40.6	43.8	34.8	33.7	29.4	36.1	34.3	34.3	30.1	31.2	27.8
34:2	14.1	14.0	19.7	21.1	27.8	28.4	25.8	24.5	26.0	24.8	25.6	22.9
34:3	0.8	1.7	1.7	2.8	1.9	3.1	3.4	2.8	1.2	2.2	1.7	2.1
36:0	–	0.1	0.1	0.04	–	0.04	0.1	0.1	0.3	0.1	0.2	0.1
36:1	3.3	3.4	1.3	1.8	2.9	2.1	1.9	1.9	2.1	2.3	2.6	3.5
36:2	14.1	10.5	9.6	8.8	8.5	8.6	7.8	7.4	9.0	9.1	8.6	10.1
36:3	0.9	4.7	4.4	7.3	7.6	9.2	6.3	6.9	7.4	8.6	7.5	9.4
36:4	–	2.4	1.0	2.8	1.9	4.3	1.4	3.3	2.3	3.4	3.5	3.8
36:5	–	0.3	0.7	0.3	1.5	0.4	1.3	0.4	1.2	0.5	0.9	0.5
38 (total)	0.9	0.4	–	–	1.5	0.4	1.3	0.4	2.0	1.1	1.9	0.9
INR	67		100		35		13		28		51	

*Control subject, controlled diet (CD).
**Type II patients, free choice diet (FC).
***Type II, patients, breakfast with lard (LD).

analyses and suggest that the calculated and determined distributions of the sn-2,3-diacylglycerols are identical. Therefore, the fatty acids have been esterified to each position of the glycerol molecule independently of the fatty acid composition of any other position. In the case of the chylomicrons (results not shown), the non-correlative esterification refers again to the molecular association of the sn-2-acylglycerols or of the 1,2-diacylglycerols and the fatty acids entering the sn-3- position (23), while in the VLDL of hepatic origin the association would concern the 1,2-diacylglycerols originating from the phosphatidic acid pathway and the free fatty acids entering the sn-3-position.

A comparison of the molecular species calculated for the sn-1,2- and sn-2,3-diacylglycerol moieties, using the 1-random 2-random and 2-random 3-random method, indicated marked differences in the proportion of the species representing the reverse isomers. These differences were seen for the VLDL and chylomicron triacylglycerols of both control subjects and patients and were a result of the overall asymmetry of the fatty acid distribution in the triacylglycerol molecules. In view of the close agreement between the determined and calculated compositions of the species by carbon and double bond number of the sn-1,2- and sn-2,3-diacylglycerols (Tables 5 and 6), it may be concluded that the calculated species provide a correct account of the molecular association and positional distribution of the fatty acids in the diacylglycerol moieties of the triacylglycerols for all samples of the control subjects and patients. These distributions allow a complete reconstitution of the original triacylglycerol composition by calculation as shown below.

Reconstitution of Molecular Species of Triacylglycerols

Since the experimental and calculated distributions for the sn-1,2- and sn-2,3-diacylglycerol moieties of the triacylglycerols agreed closely when compared on the basis of proportions of the various unsaturation classes within each carbon number, it was possible to derive a valid approximation of the true composition of the original triacylglycerols by means of the 1-random 2-random 3-random calculation (24). Table 7 gives the calculated compositions of the major molecular species of VLDL triacylglycerols from a control subject and a patient with Type II hyperlipoproteinemia. The molecular species are arranged in order of increasing double bond number within each carbon number, which are listed in order of increasing chain

TABLE 6

Molecular Species of sn-2,3-diacylglycerols Derived from VLDL Triacylglycerols of Normal Subjects and Patients with Type II Hyperlipoproteinemia

Molecular species	L1822*		L1899**		M0009**		M0010**		M0044***		M0046***	
	Exptl.	Calc.	Exptl.	Calc.	Exptl.	Calc.	Exptl.	Calc.	Exptl.	Calc.	Exptl.	Calc.
						Mole %						
30:0	0.2	0.2	0.47	0.1	–	–	0.1	0.2	0.1	0.1	0.1	0.1
30:1	0.2	0.1		0.1	0.1	0.1	0.1	0.1	–	0.0	–	0.0
32:0	1.1	1.2	0.9	1.1	0.4	0.6	0.8	0.9	0.6	0.0	1.2	0.7
32:1	2.3	1.9	1.9	1.7	0.8	0.9	2.3	1.7	1.1	1.1	1.3	1.1
32:2	1.1	0.4	0.9	0.6	0.5	0.4	0.7	0.7	0.6	0.5	0.5	0.5
34:0	–	0.7	–	0.4	0.3	0.3	–	0.4	0.4	0.3	1.1	0.1
34:1	15.3	13.8	12.7	11.3	7.7	7.5	11.1	9.7	8.6	8.8	10.6	10.5
34:2	9.3	8.0	11.0	10.7	9.3	8.2	9.6	9.5	6.3	7.8	6.2	9.2
34:3	0.8	1.1	2.1	2.6	1.4	2.1	1.4	2.5	1.8	1.9	2.8	2.1
34:4	–	0.2	–	0.1	–	–	–	–	–	–	–	–
36:0	0.1	0.1	–	0.03	–	0.02	0.1	0.03	0.1	0.0	0.6	0.6
36:1	1.3	3.6	3.4	1.7	1.3	1.5	1.2	1.3	1.2	1.4	1.9	2.2
36:2	45.3	39.9	36.2	29.2	26.0	24.1	26.4	26.5	27.1	26.7	23.0	24.3
36:3	13.5	18.5	21.2	27.2	31.3	34.5	26.8	29.1	33.4	33.2	31.4	31.4
36:4	5.3	4.4	3.5	8.0	10.4	14.0	10.0	10.1	9.5	11.6	9.9	11.9
36:5	0.5	0.6	–	0.7	4.2	1.2	2.2	1.0	3.1	0.9	3.5	0.9
38 (total)			4.5	2.3	4.9	2.7	5.3	3.1	5.3	1.2	4.7	2.0
INR	66		116		42		15		29		30	

*Control subject, controlled diet (CD).
**Type II patients, free choice diet (FC).
***Type II, patients, breakfast with lard (LD).

length. Table 7 includes only the species contributing more than 0.5% of the total VLDL-TG in at least one of the samples. The 36 species identified account for about 80% of the total triacylglycerol mass of VLDL in each instance. It is interesting to note that 16:0 18:1 18:1 species make up about 25% and 17% of the total in the control subject and patient, respectively, and that it is essentially one and the same enantiomer type in both instances. Likewise, the 16:0 18:2 18:1 species, which is present to the extent of 6% and 11% in the control subject and the patient, respectively, is also essentially one enantiomer. Therefore, the marked asymmetry of the VLDL triacylglycerols is due largely to the asymmetry of a few major triacylglycerols.

DISCUSSION

Validity of Calculation of Molecular Species of Triacylglycerols

The calculation of the molecular species of triacylglycerols on the basis of the 1-random 2-random 3-random distribution is justified by the experimental demonstration of the existence of a non-correlative distribution of fatty acids in the sn-1,2- and sn-2,3-diacylglycerol moieties of the original triacylglycerols. A further experimental validation of the calculated distribution is provided by the close agreement between the calculated and the determined composition of the sn-1,3-diacylglycerols, and the close agreement between the triacylglycerol compositions calculated from the sn-1,2-, sn-2,3- and sn-1,3-diacylglycerol randomization with the corresponding compositions of the fatty acids in the sn-1-, sn-3 and sn-2-positions (6).

The 1-random 2-random 3-random distribution is consistent with certain metabolic facts about triacylglycerol biosynthesis and secretion by animal and human liver. Thus, it is known that acyltransferases exist, which are specific for the sn-1- and sn-2-positions of sn-glycerol-3-phosphate leading to the formation of phosphatidic acids with distinctly different complements of fatty acids in these positions (25,26). Furthermore, final acylation of the sn-3-position occurs independently of the fatty acid composition of either the sn-1- or the sn-2-position. It is also well known that the sn-3-position of the triacylglycerol molecule is synthesized last and that a fatty acid pool different from that involved in the synthesis of the

TABLE 7

Distribution of Molecular Species of Triacylglycerols in VLDL from a Normal Subject and a Patient with Type II Hyperlipoproteinemia

Carbon number	Molecular species*			Control subject (L1822 CD)	Type II patient (M0010 FC)
				Moles %	
48:0	16:0	16:0	16:0	0.737	0.604
50:1	16:0	16:0	18:1	5.340	2.910
50:1	16:0	18:1	16:0	3.495	3.599
50:2	14:0	18:1	18:1	1.204	0.814
50:2	16:0	16:0	18:2	1.200	1.498
50:2	16:0	16:1	18:1	1.993	1.838
50:2	16:0	18:1	16:1	1.143	0.880
50:2	16:0	18:2	16:0	0.859	2.252
50:3	14:0	18:2	18:1	0.296	0.509
50:3	16:0	16:1	18:2	0.448	0.946
52:1	16:0	18:0	18:1	1.162	0.644
52:1	16:0	18:1	18:0	1.618	0.931
52:2	16:0	18:1	18:1	25.327	17.336
52:2	18:1	16:0	18:1	1.351	0.550
52:2	18:1	18:1	16:0	0.884	0.681
52:2	16:0	18:2	18:0	0.398	0.582
52:3	16:0	18:1	18:2	5.695	8.926
52:3	16:0	18:2	18:1	6.225	10.846
52:3	16:1	18:1	18:1	2.226	1.422
52:3	18:1	16:1	18:1	0.504	0.348
52:4	16:0	18:1	18:3	0.565	0.474
52:4	16:0	18:2	18:2	1.399	5.585
52:4	16:1	18:1	18:2	0.500	0.732
52:4	16:1	18:2	18:1	0.547	0.889
54:2	18:0	18:1	18:1	2.206	0.960
54:2	16:0	18:1	20:1	0.565	0.474
54:3	18:0	18:2	18:1	0.542	0.600
54:3	18:1	18:1	18:1	6.411	3.280
54:4	18:1	18:1	18:2	1.441	1.689
54:4	18:1	18:2	18:1	1.575	2.052
54:4	18:2	18:1	18:1	1.468	1.417
54:5	16:0	20:4	18:1	0.504	0.487
54:5	16:0	18:1	20:4	0.251	0.520
54.5	18:1	18:2	18:2	0.354	1.056
54:5	18:2	18:1	18:2	0.330	0.729
54:5	18:2	18:2	18:1	0.361	0.886

*Positions of enantiomeric triacylglycerols read from left to right as follows: sn-1-, sn-2- and sn-3-position.

phosphatidic acid is utilized (27). Previously, a non-correlative esterification of fatty acids in the various glycerol positions had been claimed by Slakey and Lands (28) for total rat liver triacylglycerols, which possess fatty acid compositions closely similar to those seen in the VLDL and chylomicrons of man. Interestingly, Wood and Harlow (29) could not confirm the findings of Slakey and Lands with rat liver triacylglycerols, although a relationship of this kind was found in tumor triacylglycerols (30). In contrast, Christie and Moore (31) obtained excellent agreement for a variety of pig tissues, except the blood triacylglycerols. We also have shown a non-correlative distribution of fatty acids for lard (13).

Effect of Diet on Triacylglycerol Structure

The structural analyses of the VLDL triacylglycerols were performed on one or more normolipemic subjects and one or more Type II hyperlipoproteinemia patients after 2 weeks on a free choice diet (Visit 1), after 2 weeks on a standardized diet excluding lard (Visit 4) and 4 hr after a breakfast containing lard (Visit 5). In all instances the triacylglycerol composition was clearly consistent with a non-correlative distribution of fatty acids. After the lard meal,

there was relatively more palmitic acid in the sn-2-position and stearic acid in the sn-3-position. Since lard contains a high proportion of palmitic acid in the sn-2-position and since this position is believed to survive intestinal lipolysis, it would be anticipated that increased proportions of palmitate in the sn-2-position of the chylomicron triacylglycerols would be found. The proportion of total palmitate in the sn-2-position increased from about 10% to nearly 40% after the lard meal. However, the bulk of palmitate was still present in the sn-1-position. As a result relatively more oleate and linoleate appeared in the sn-1-position, but the bulk of these acids again was confined to the sn-2- and sn-3-positions. On the basis of the present results it is obvious that the composition and positional distribution of fatty acids in the VLDL and chylomicron triacylglycerols is affected by both the composition and positional distribution of the fatty acids in the diet. The molecular association, however, remained characteristic of the 1-random 2-random 3-random distribution.

Triacylglycerol Differences Among Normal Subjects and Patients with Type II Hyperlipoproteinemia

Theoretically, the VLDL triacylglycerols of the control subjects and Type II patients subsisting on free choice diets could have differed in the overall composition of the fatty acids, in the positional and stereospecific placement of the fatty acids and in their molecular association. These differences could have occurred because of discrepancies in the uptake and utilization of the individual dietary fatty acids and monoacylglycerols, and in the relative levels and activity of different acyltransferases, and the relative overall activity of the different metabolic pathways contributing to the biosynthesis of the triacylglycerols in the intestine and in the liver (32). For similar reasons, there could have been differences in the composition, positional distribution and molecular association of the fatty acids in the chylomicron triacylglycerols between the normal subjects and Type II patients, although chylomicrons are produced only in the intestinal mucosa. In addition, differences in the composition, positional distribution and even molecular association of the fatty acids of the VLDL triacylglycerols could have resulted from differences in the positional and fatty acid specificity of lipoprotein and hepatic lipase, which convert the chylomicrons into VLDL-like lipoproteins, and VLDL into LDL-like or remnant particles (33-35). As a result characteristic differences could have been expected in the structure of plasma triacylglycerols as already reported by Gordon et al. (3) for Type II patients and by Parijs et al. (2,4) for Type IV patients. The present results and the results of our previous study (6), however, fail to establish the anticipated differences or to confirm the differences claimed by previous investigators to exist between normal subjects and patients with hyperlipemia in the structure of their VLDL and chylomicron triacylglycerols. These findings tend to refute the possibility that differences exist in the relative positional placement and molecular association of fatty acids of plasma triacylglycerols between normolipemic and hyperlipemic individuals.

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Dealkylation of Various 24-Alkylsterols by the Nematode *Caenorhabditis elegans*

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ABSTRACT

The metabolism of 4 dietary 24-alkylsterols was investigated in the free-living nematode *Caenorhabditis elegans.* The major unesterified sterols of *C. elegans* in media supplemented with either campesterol, 22-dihydrobrassicasterol or stigmasterol included cholesta-5,7-dienol, cholesterol, cholest-7-enol, and 4α-methylcholest-8(14)-enol. Dietary stigmastanol yielded cholest-7-enol, cholestanol, cholest-8(14)-enol, and 4α-methylcholest-8(14)-enol as major unesterified sterols. Esterified sterols comprised less than 22% of the total sterol. Removal of a C-24 ethyl substituent of sterols was neither hindered by the presence of a Δ^{22}-bond in the sterol side chain nor was it dependent on unsaturation in ring B of the steroid nucleus. *C. elegans* reduced a Δ^{22}-bond during its metabolism of stigmasterol; it did not introduce a Δ^{5}-bond during stigmastanol metabolism. *C. elegans* was capable of removing a C-24 methyl substituent regardless of its stereochemical orientation. Metabolic processes involving the steroid ring system of cholesterol (C-7 dehydrogenation, Δ^{5}-reduction, 4α-methylation, $\Delta^{8(14)}$-isomerization) in *C. elegans* were not hindered by the presence of a 24-methyl group; various 24-methylsterol metabolites from campesterol were detected, mostly 24-methylcholesta-5,7-dienol. In contrast, no 24-ethylsterol metabolites from the dietary ethylsterols were found. More dietary 24-methylsterol remained unmetabolized than did dietary 24-ethylsterol. A 24α-ethyl group and a 24β-methyl group were dealkylated to a greater extent by *C. elegans* than was a 24α-methyl group, perhaps reflecting the substrate specificity of the dealkylation enzyme system, or suggesting different enzymes altogether.
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INTRODUCTION

A structure-function relationship appears to exist between sterols lacking a 24-alkyl substituent and their occurrence in animals possessing well-defined nervous systems (1,2). In nervous tissue, these 24-desalkylsterols function as membranous components. Although plants and fungi are able to alkylate sterols at C-24, it is generally accepted that animals do not possess a C-24 alkylation mechanism. Animals that can biosynthesize sterols de novo produce solely 24-desalkylsterols, whereas most of those incapable of de novo sterol biosynthesis exhibit a preference for 24-desalkylsterols. Such preference is demonstrated either by an absolute nutritional requirement for 24-desalkylsterols, selective uptake from an exogenous sterol mixture, or an ability to dealkylate 24-alkylsterols.

The conversion of exogenous 24-alkylsterols to cholesterol and/or other 24-desalkylsterol metabolites is found in a number of invertebrate animals (3). These include the sea anemone *Calliactis parasitica* and the oyster *Ostrea gryphea* (4), the snail *Omphalius pfeifferi* (5), many but not all insects (6-8), and 2 free-living nematodes, *Turbatrix aceti* (9) and *Caenorhabditis elegans* (10-12). Sterol dealkylation at C-24 also occurs in the protozoan *Tetrahymena pyriformis* (13).

C. elegans has been utilized widely as a model for studies of aging, genetics, embryological development and biochemistry. Like many invertebrates, nematodes are incapable of biosynthesizing sterols de novo (9,14-17) and possess a nutritional requirement for sterol (18-20). Both *C. elegans* and *T. aceti* are capable of removing the C-24 ethyl group of sitosterol (24α-ethylcholest-5-en-3β-ol) (9-12). This ability does not extend to all nematodes; the animal parasite *Ascaris lumbricoides* did not metabolize injected sitosterol (15).

It is unknown whether *C. elegans* can dealkylate sterols that differ from sitosterol in their C-24 alkyl group structure or stereochemical orientation or in their nuclear or side chain unsaturation. In the present study, *C. elegans* was propagated in media individually supplemented with a variety of sterols differing from sitosterol in one of the above structural features. The resulting sterol compositions of the harvested nematodes were identified to determine the extent of metabolism of these 24-alkylsterols by *C. elegans.*

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MATERIALS AND METHODS

C. elegans was cultured axenically in a semi-defined aqueous medium as previously described (12) and supplemented with sterol at a concentration of 25 ppm. The dietary sterols were: campesterol (24α-methylcholest-5-en-3β-ol), 22-dihydrobrassicasterol (24β-methylcholest-5-en-3β-ol), stigmasterol (24α-ethylcholesta-5,22E-dien-3β-ol), and stigmastanol (24α-ethyl-5α-cholestan-3β-ol). The purity of each sterol was greater than 98% by gas liquid chromatography (GLC). NMR analysis indicated that dihydrobrassicasterol contained ca 25% of the 24α-epimer campesterol (21).

Lipid extraction of lyophilized nematodes harvested after 2 weeks (1-2 g dry wt) was performed as before (12). Steryl esters were separated from free sterols by chromatography of the neutral lipid fraction (ca 90 mg) on a 13.0 g column of silica gel 60 using the following elution scheme: a) 100 ml 10% benzene in hexane; b) 50 ml 50% benzene in hexane; c) 100 ml benzene; d) 50 ml chloroform; e) 50 ml diethyl ether; f) 50 ml methanol. Fractions were monitored by thin-layer chromatography in 2 solvent systems of hexane/diethyl ether/acetic acid: S_1 (80/20/1) and S_2 (98/2/1) using standards of cholesterol (R_f:S_1, 0.10; S_2, 0.03), cholesteryl acetate (S_1, 0.50; S_2, 0.18), and cholesteryl oleate (S_1, 0.68; S_2, 0.38). The steryl ester fraction (b+c) and the free sterol fraction (e) each were saponified in 5% methanolic KOH for 3 hr at 70 C followed by isolation procedures previously described (12): separation of 4-methylsterol and 4-desmethylsterol fractions, acetylation and separation of steryl acetates by argentation column chromatography.

Quantitative analyses were accomplished by GLC. Final identifications were made by GLC, gas chromatography-mass spectrometry (GC-MS) and, for certain samples, UV spectroscopy (of steryl acetates in hexane). GLC of free sterols and steryl acetates was performed using 2 chromatographic systems: a coiled glass column (2 m x 2 mm ID) packed with 2.0% OV-17 (50% phenyl-50% methyl silicone) at 250 C and a fused silica capillary column (13.8 m x 0.25 mm ID) with a 0.25 μm film of DB-1 (bonded methyl silicone) at 240 C. GC-MS was carried out on a Finnigan model 4510 instrument equipped with a 15 m x 0.32 mm DB-1 capillary column (0.25 μm film). Other instrumental details have been supplied previously (12).

RESULTS

Growth of *C. elegans* was neither stimulated

TABLE 3

GLC Analysis of Sterols from *Caenorhabditis elegans*

Sterol acetate	RRT[a] DB-1	RRT[a] OV-17
Cholesta-5,7,9(11)-trienol	0.98	1.06
Cholest-8(14)-enol	0.99	1.02
Cholesterol	1.00	1.00
Cholestanol	1.02	1.02
Cholesta-5,7-dienol	1.10	1.17
Cholest-7-enol	1.13	1.18
24-Methylcholesta-5,7,9(11)-trienol	1.26	1.40
24-Methylenecholesterol	1.27	1.35
24-Methylcholesterol	1.29	1.32
24-Methylenecholesta-5.7-dienol	1.40	1.56
24-Methylcholesta-5,7-dienol	1.43	1.53
Stigmasterol	1.40	1.44
24-Methylcholest-7-enol	1.46	1.56
Stigmastanol	1.65	1.66
4α-Methylcholest-8(14)-enol	1.14 (1.17)[b]	1.13 (1.16)[b]
4α-Methylcholest-7-enol	1.27 (1.31)[b]	1.31 (1.35)[b]
4α,24-Dimethylcholest-8(14)-enol	1.47 (1.51)[b]	1.48 (1.52)[b]
4α,24-Dimethylcholestanol	1.51 (1.55)[b]	1.48 (1.52)[b]

[a]RRT = Retention time of sterols as acetate derivatives relative to cholesteryl acetate under chromatographic conditions as described in Experimental Conditions.

[b]Retention time of sterols as free alcohol relative to cholesterol.

TABLE 1

Lipid and Sterol Content of *Caenorhabditis elegans* Propagated with Different Dietary Sterols[a]

Dietary sterol	Total lipid (% of dry wt)	Total sterol (% of dry wt)	Esterified sterol (% of total sterol)
Campesterol	18.7	0.12	15.9
Dihydrobrassicasterol	18.4	0.10	21.3
Stigmasterol	19.0	0.11	17.1
Stigmastanol	18.7	0.14	7.3
Sitosterol	18.3	0.13	13.3

[a]Sitosterol data were reported previously (11).

TABLE 2

Sterol Composition (as relative percentages) of Free Sterol (FS) and Steryl Ester (SE) Fractions from *C. elegans* Propagated with Different Dietary Sterols[a]

	Dietary sterol[b]									
	Campesterol		Dihydro-brassicasterol		Stigmasterol		Stigmastanol		Sitosterol	
Recovered sterol	FS	SE	FS	SE	FS	SE	FS	SE	FS	SE
Unmetabolized dietary sterol	35.8	53.3	24.2	31.7	20.5	21.9	14.2	30.1	16.7	31.6
Cholesta-5,7-dienol	29.4	10.9	45.0	21.5	55.6	26.5	–	–	66.5	30.5
Cholesterol	3.9	3.7	5.1	8.5	8.6	11.3	–	–	6.7	9.3
Cholest-7-enol	3.7	1.8	3.5	1.6	3.9	9.4	68.3	28.6	4.4	3.6
Cholestanol	–	–	–	–	–	–	3.8	5.6	–	–
Cholest-8(14)-enol	–	–	–	–	–	–	3.7	6.4	–	–
Cholesta-5,7,9(11)-trienol	1.9	–	6.5	3.4	5.8	2.4	–	–	0.8	0.3
4α-Methylcholest-8(14)-enol	3.6	9.7	6.7	14.7	5.4	27.5	9.3	27.0	4.2	23.3
4α-Methylcholest-7-enol	0.2	0.2	0.3	0.6	0.2	1.0	0.7	2.3	0.7	1.4
24-Methylcholesta-5,7-dienol	14.1	12.9	5.4	7.1	–	–	–	–	–	–
24-Methylcholest-7-enol	0.6	1.1	0.2	–	–	–	–	–	–	–
24-Methylenecholesterol	3.1	3.7	1.3	8.8	–	–	–	–	–	–
24-Methylenecholesta-5,7-dienol	1.3	–	0.3	–	–	–	–	–	–	–
24-Methylcholesta-5,7,9(11),trienol	1.0	0.3	1.1	1.3	–	–	–	–	–	–
4α,24-Dimethylcholest-8(14)-enol	0.7	1.4	0.3	0.4	–	–	–	–	–	–
4α,24-Dimethylcholestanol	0.4	0.8	0.1	0.4	–	–	–	–	–	–
Fucosterol	–	–	–	–	–	–	–	–	0.1	0.1
24-Desalkylsterol metabolites	42.7	26.3	67.1	50.3	79.5	78.1	85.8	69.9	83.3	68.4
24-Alkylsterol metabolites	21.2	20.2	8.7	18.0	–	–	–	–	0.1	0.1

[a]Dashed line (–) indicates sterol not detected.

[b]Sitosterol data were reported previously (11).

nor inhibited by the presence of any of the 4 dietary sterols, relative to its growth in sitosterol-supplemented media. Total lipid and sterol content values (Table 1) showed little deviation, regardless of the dietary sterol. In all cases, steryl esters were present, but the recovered sterols were mostly nonesterified; free sterols comprised 79-93% of the total sterol (Table 1). Stigmastanol yielded less esterified sterol than did the other dietary sterols.

The sterol compositions of free sterol and steryl ester fractions obtained from *C. elegans* propagated with each of the 4 dietary sterols are shown in Table 2. Identification of sterols from *C. elegans* was made on the basis of their GLC relative retention time (RRT) values (Table 3), both as free sterols and as steryl acetates, and confirmed by GC-MS. All data were in agreement with those of authentic sterol standards, with the exception of the following, for which no reference compounds were available: 24-methylcholesta-5,7,9(11)-trienol, 24-methylenecholesta-5,7-dienol, 4α,24-dimethylcholest-8(14)-enol, and 4α,24-dimethylcholestanol. These 4 sterols previously have not been detected in nematodes. However, their experimental RRTs agreed with their calculated values, GC-MS analyses yielded fragmentation patterns expected of the proposed structures and UV spectroscopy of the conjugated diene and triene confirmed their identification.

The mass spectrum of 24-methylenecholesta-5,7-dienyl acetate included the following significant ions [m/z (fragment, relative intensity)]: 438 (M^+, 1%), 378 (M^+-HOAc, 45), 363 (M^+-HOAc-CH_3, 6), 253 (M^+-HOAc-side chain, 8), 227 (M^+-HOAc-side chain-C_2H_2, 1), 211 (M^+-HOAc-side chain-C_3H_6, 5), 195 ($C_{15}H_{15}$, 3), 158 ($C_{12}H_{14}$, 30), 143 ($C_{11}H_{11}$, 29), and 55 (C_4H_7, 100). Ions corresponding to these fragments were produced by 24-methylcholesta-5,7,9(11)-trienyl acetate at 438 (5%), 378 (100%), 363 (23%), 251 (27%), 225 (13%), 209 (64%), 195 (53%), 158 (14%), 143 (19%), and 55 (99%). As for the pair of 4,24-dimethylsterols, characteristic ions were observed in the mass spectrum of 4,24-dimethylcholest-8(14)-enol: 414 (M^+, 63%), 399 (M^+-CH_3, 10), 381 (M-H_2O-CH_3, 1), 287 (M^+-side chain, 9), 269 (M^+-H_2O-side chain, 4), 245 (M^+-side chain-C_3H_6, 3), 243 (M^+-H_2O-side chain-C_2H_2, 6), 227 (M^+-H_2O-side chain-C_3H_6, 8), and 55 (C_4H_7, 100). Correspondingly, 4,24-dimethylcholestanol produced the following ions: 416 (21%), 401 (7%), 383 (3%), 289 (1%), 271 (0%), 247 (18%), 245 (1%), 229 (31%), and 55 (100%).

The GLC RRTs (Table 3) of the 4 4-methylsterols as free alcohols relative to cholesterol were 0.03-0.04 units greater (ca 3% greater)

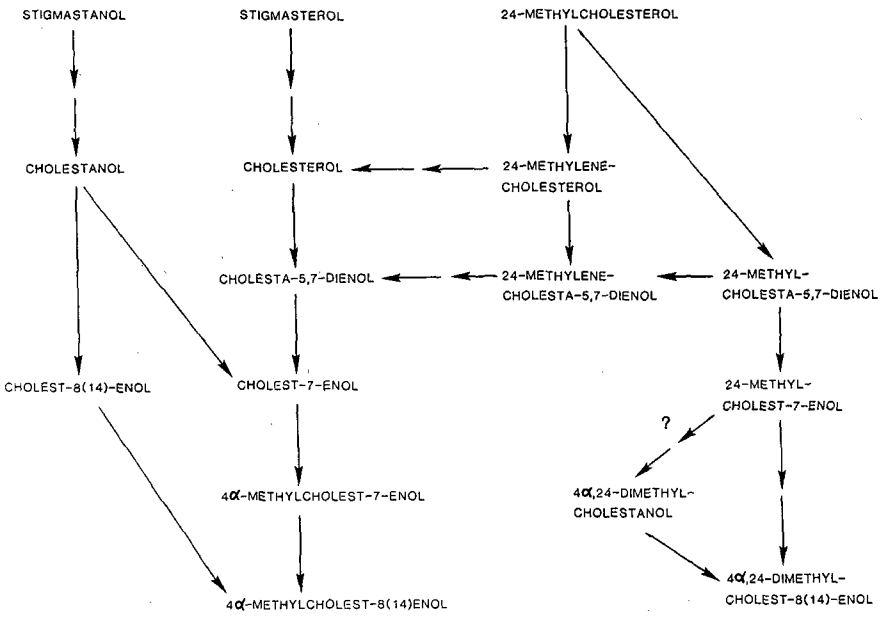

FIG. 1. Hypothetical pathways of sterol metabolism in *C. elegans.*

than their corresponding acetate RRTs relative to cholesteryl acetate. Such an increase is characteristic of 4-methylsterols (22).

UV spectra of the $\Delta^{5,7}$-diene sterols (cholesta-5,7-dienol, 24-methylcholesta-5,7-dienol, 24-methylenecholesta-5,7-dienol) exhibited typical absorption at λ_{max} 262, 270, 280, 292 nm; the $\Delta^{5,7,9(11)}$-trienols (cholesta-5,7,9(11)-trienol and 24-methylcholesta-5,7,9(11)-trienol) absorbed characteristically (23) at λ_{max} 308, 322, 338 nm.

DISCUSSION

Dietary stigmasterol was metabolized by *C. elegans* to yield the same sterol metabolites in approximately the same relative percentages as those obtained previously from dietary sitosterol (11,12). All of the detected metabolites (ca. 80% of total recovered sterol) of stigmasterol were 24-desalkylsterols, predominantly cholesta-5,7-dienol, indicating that *C. elegans* is capable of dealkylating stigmasterol. Metabolism of stigmasterol likely occurs in the sequence shown in Figure 1. Removal of the 24α-ethyl group is therefore not hindered by the presence of a Δ^{22}-bond in the sterol side chain. *C. elegans* is capable of Δ^{22}-reduction, since no Δ^{22}-sterol metabolites were detected. Dealkylation either proceeds in the presence of the Δ^{22}-bond, after which the double bond is reduced, or else dealkylation occurs after Δ^{22}-reduction. Unpublished studies, in which we have identified Δ^{22}-24-desalkylsterols (but not 22-dihydro-24-alkylsterols) in cultures grown in media containing stigmasterol plus a dealkylation inhibitor, suggest that dealkylation proceeds in the presence of the Δ^{22}-bond which subsequently is reduced. The tobacco hornworm, *Manduca sexta,* similarly dealkylates stigmasterol to produce cholesterol by first removing the C-24 ethyl substituent to yield a $\Delta^{22,24}$ side chain, and then reducing the Δ^{22}-bond (24). A $\Delta^{22,24}$-sterol substrate is essential for Δ^{22}-reduction to occur in this insect, as dietary 22-dehydrocholesterol was not converted to cholesterol (25). Possibly, the Δ^{22}-reductase in *C. elegans* might also require a $\Delta^{22,24}$-sterol substrate.

Since *C. elegans* could dealkylate sitosterol and stigmasterol, both of which contain a Δ^5-bond in ring B, it was of interest to determine whether ring B unsaturation (or lack of it) affected C-24 dealkylation in the sterol side chain. From our results, stigmastanol, the saturated analog of stigmasterol and sitosterol, also was dealkylated and was further metabolized to produce mostly cholest-7-enol. Although cholesta-5,7-dienol is the predominant metabolite of dietary Δ^5-sterols, no Δ^5- or $\Delta^{5,7}$-sterols were produced from stigmastanol. *C. elegans* thus may lack a Δ^5-dehydrogenase. Figure 1 illustrates 2 possible metabolic pathways originating from stigmastanol. Presumably, cholestanol is an intermediate from the 24-ethylstanol to cholest-7-enol. Studies (unpublished results) utilizing cholestanol as dietary sterol support its metabolic conversion as proposed here. Thus, removal of the 24α-ethyl

group proceeded in the presence of a saturated steroid nucleus and is probably independent of ring B unsaturation. Metabolism of stigmastanol in *C. elegans* is similar to that exhibited by the Mexican bean beetle, *Epilachna varivestis* (26). This phytophagous insect reduces Δ^5-24-ethylsterols to stigmastanol; as in *C. elegans*, the ethylstanol is then dealkylated to cholestanol, which is converted to cholest-7-enol.

With regard to the 24-methylsterols, metabolism of both epimers by *C. elegans* occurred, but in a manner different from its metabolism of 24-ethylsterols, as proposed in Figure 1. Both 24-methylsterols were dealkylated and then metabolized within the ring nucleus in the same manner as the dealkylated 24-ethylsterols. However, a significant portion (ca 20%) of dietary campesterol remained alkylated, yet modifications to the ring system of campesterol proceeded as usual (Δ^7-dehydrogenation, 4α-methylation, etc.) to form several 24-methylsterol metabolites, predominantly 24-methylcholesta-5,7-dienol (Table 2). The sterol composition obtained from dihydrobrassicasterol is equivocal due to the substantial campesterol impurity in the diet; the 24-methylsterol metabolites (9% of free sterol) may have originated from the campesterol rather than the 24β-epimer. Nevertheless, this percentage of 24-methylsterol metabolites was less than that obtained from the stereochemically pure campesterol, indicating that more dihydrobrassicasterol than campesterol was dealkylated. In contrast, the 24-ethylsterol substrates yielded no detectable 24-ethyl-(or methyl-) sterol metabolites. Much more dietary 24α-methylsterol remained unmetabolized than did dietary 24α-ethylsterol (Table 2). Altogether, 24α-ethyl and 24β-methyl groups were dealkylated to a greater extent than was a 24α-methyl group. These findings could reflect substrate specificity of the dealkylation enzyme system whereby 24α-ethyl and 24β-methyl groups are more efficiently removed than a 24α-methyl group. Alternatively, 2 enzymes may function according to the size of the 24-alkyl substrate: one highly efficient system which catalyzes dealkylation of 24-ethylsterols, and another enzyme that dealkylates C-24 methyl groups and for which a 24β-methylsterol provides a better substrate than a 24α-methylsterol. Yet another pair of enzyme systems might exist, based on the stereochemical orientation of the 24-alkyl substrate: one enzyme removing only α-alkyl groups for which ethylsterols are better substrates than methylsterols, and another enzyme dealkylating only sterols with β-alkyl groups.

The nuclear modifications (C-7 dehydrogenation, Δ^5-reduction, 4α-methylation, $\Delta^{8(14)}$-isomerization) which occur to cholesterol in *C. elegans* (11) also occurred to 24-methylcholesterol. Thus, metabolism of the sterol ring system was not hindered by the presence of a 24-methyl group. Possibly, a 24-ethyl substituent abolishes any affinity for binding between the sterol substrate and the enzyme(s) for nuclear metabolism, whereas a 24-methylsterol substrate still retains some affinity. However, 4α,24-dimethylcholest-7-enol (corresponding to 4α-methylcholest-7-enol which was present) was not discovered, nor was 4α-methylcholestanol (corresponding to 4α,24-dimethylcholestanol) found. Possibly, these 2 sterols may have been present but quantitatively insufficient for detection.

The occurrence of the $\Delta^{24(28)}$-sterol 24-methylenecholesterol indicates its intermediate role in the dealkylation of 24-methylcholesterol. If so, the C-24 demethylation mechanism in *C. elegans* is perhaps similar to that in insects which also proceeds via a $\Delta^{24(28)}$-methylenesterol (27). It previously has been demonstrated that the pathway of 24-ethylsterol (sitosterol) dealkylation in *C. elegans* shares similarities with that process in insects, such as the intermediacy of a $\Delta^{24(28)}$-ethylidenesterol, fucosterol (11,12).

The steryl ester compositions generally were similar to the corresponding free sterol compositions, with some notable exceptions. The predominant sterol metabolite in each case (either cholesta-5,7-dienol or cholest-7-enol) comprised a percentage of the free sterol fraction which was more than twice its percentage of the steryl esters. Unmetabolized dietary sterol generally formed a greater proportion of the ester fraction than of the free sterols; an ability of *C. elegans* to sequester or store unmetabolized 24-alkylsterols as steryl esters was suggested previously (11). The ester fraction also contained a much greater proportion of 4α-methylcholest-8(14)-enol than did the free sterol fraction, a finding we also observed using other dietary sterols (11). Steryl esters are considered to function primarily as storage forms of free sterol, to be hydrolyzed as requirements for free sterol arise. Based upon the propensity of *C. elegans* to biosynthesize and esterify 4α-methylcholest-8(14)-enol, this sterol may indeed function in some as yet unknown, specialized capacity as a steryl ester, rather than fulfill the general role of esters as storage products.

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Lipid Analyses of Isolated Surface Membranes of *Leishmania Donovani* Promastigotes

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ABSTRACT

Constituent lipids of surface membranes (SM) isolated from *Leishmania donovani* promastigotes were analyzed and compared with those obtained from whole cells and an isolated kinetoplast-mitochondrion fraction (KM). On a dry weight basis, the total extractable lipids constituted ∿47%, 12% and 24% of the SM, cells and KM, respectively. The total lipids of SM, cells and KM all were composed of ∿70% phospholipids (PL), 20-25% neutral lipids and 5-10% glycolipids. Sterols and diglycerides composed 60% and 30%, respectively, of the various neutral lipid fractions. Several mannose- and galactose-containing glycolipids were fractionated but not identified. The glycolipid fractions from cells and SM had demonstrable antigenic activities with rabbit anti-SM sera. Striking quantitative differences were apparent between the PL profiles of the 3 cellular components examined. The PL of SM, whole cells and KM, respectively, were composed of: 15%, 51% and 24% phosphatidylcholine; 37%, 13% and 11% phosphatidylethanolamine (PE); 18%, 10% and 14% phosphatidylinositol; 10%, 1% and 4% phosphatidylserine and traces of cardiolipin, phosphatidylglycerol and phosphatidic acid. An unknown PL containing sphingosine, choline and vicinal hydroxyl groups but no free amino moieties made up ∿19% of the PL of SM and whole cells, but it constituted ∿27% of the PL of KM. The PL side chain constituents of whole cells and SM were composed mainly of longchain fatty acids (C18-20). Further, over 50% of the PE of SM was in the alkyl and alK-1-enyl ether forms. These SM properties might contribute to the organism's resistance to digestion in the hydrolytic environs of both its insect vector and mammalian hosts.
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INTRODUCTION

The protozoan parasite *Leishmania donovani* has a digenetic life cycle which includes an extracellular flagellated promastigote stage in the alimentary tract of its insect vector and an obligate intracellular amastigote stage within the lysosomal system of mammalian macrophages (1-4). This life cycle is sustained by a number of phenomena that presumably are dependent upon mediation by the parasite plasma membrane. Therefore, examination of the biochemical and physiological properties of the parasite surface membrane may elucidate the mechanisms which ultimately lead to host infection.

Purified surface membranes (SM) from *L. donovani* promastigotes were isolated first in this laboratory and demonstrated to consist of a stable structural complex of the organism's plasma membrane with subpellicular microtubules attached to their inner (cytoplasmic) surface (5,6). Using such isolated preparations, we demonstrated the net negative surface charge of SM and the uniform external distribution of carbohydrate ligands on them (6). Further, the SM were shown to contain ∿23 iodinable mannose-containing glycoproteins and other glycoconjugates (7,8). Moreover, using purified SM we have identified and characterized an unusual externally oriented membrane-bound acid phosphatase (9-11), 2 separate (a 5′- and a 3′-) nucleotidase activities (12,13) and 3 distinct lipolytic enzymes (phospholipases A_1, A_2 and C) (manuscript in preparation).

Although it is well recognized that lipids are an integral part of cellular membranes, to date the lipid composition of the *Leishmania* promastigote SM has remained unidentified. Therefore, the current study was undertaken to determine the constituent lipids of SM isolated from *L. donovani* promastigotes. For comparative purposes, lipid analyses also were performed with whole *L. donovani* promastigotes and kinetoplast-mitochondria isolated from such cells.

MATERIALS AND METHODS

Leishmania donovani strain 1-S, clone 2D promastigotes (14) were maintained and grown in Steiger and Black's chemically defined RE-IX

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medium (15) at 26 C. Mid- to late-log phase cells (3-6 x 10^7/ml) were harvested by centrifugation at 6,000 x g for 10 min at 4 C. The cells were resuspended and washed once in ice-cold Hanks' balanced salt solution (GIBCO, Grand Island, New York) and subsequently 3 times in Tris-buffered saline (10 mM Tris-HCl, 145 mM NaCl, pH 7.4). Total lipids were extracted from whole washed cells suspended in Tris-buffered NaCl. Surface membranes (SM) and kinetoplast-mitochondrion (KM) fractions were isolated from whole cells according to the method of Dwyer (5) as modified by Gottlieb and Dwyer (10). The purity/enrichment of these fractions was assessed as previously described (5,10). The isolated subcellular fractions were washed twice in 20 mM Tris-HCl, 3 mM $MgCl_2$, 0.25 M sucrose buffer, pH 8.0 by centrifugation at 48,000 x g for 30 min at 4 C. Subsequently, they were resuspended in the Tris-NaCl buffer above prior to lipid extraction.

Lipids were extracted by adding a volume of chloroform and 2 volumes of methanol (1:2, v/v) to a volume of either whole cells, isolated SM or KM in buffer above (16). These mixtures were sonicated in an ice bath for a total of 5 min with intermittent high speed vortex mixing for 1 min. Subsequently, these samples were centrifuged at 6000 x g for 5 min at 4 C. The organic phase was removed and the pelleted material was exhaustively reextracted by sonication and centrifugation in sequentially increasing concentrations of chloroform in methanol and finally in chloroform. The combined supernatants were concentrated in vaccuo to ~1.5-2 ml and freed of non-lipid contaminants via passage through a Sephadex G-25 column (17). Subsequently, the total lipid extracts were fractionated according to their polarity using silicic acid column chromatography as described by Rouser et al. (17).

Quantitative analyses of individual phospho- and glycolipid components in fractions obtained from silicic acid column chromatography were made by separation on 2-dimensional thin layer chromatography (TLC) plates (Supelco, Inc., Bellefonte, Pennsylvania) (17). Individual lipid spots were analyzed for phosphorus (18) and sugar (19) contents. Ester bond and plasmalogen contents were assessed on the TLC-separated components using the procedures of Ferrel et al. (20) and of Renkonen (21), respectively, as described by Kates (19). Neutral lipids were subfractionated by TLC on silica gel-HR thin layer plates (Analtech Inc., Newark, Delaware) using a hexane/ether/glacial acetic acid (40:60:1, v/v/v) solvent system and visualized by charring at 105 C for 3 min (22).

Individual phospholipid components, separated by 2-dimensional TLC and visualized by exposure to iodine vapor, were transmethylated (23) and the resulting fatty acid methyl esters were analyzed by gas-liquid chromatography (GLC). Analyses were done using a Varian gas chromatograph (Model 3700, Varian Instruments, Palo Alto, California) equipped with a Varian CDS-111 electronic integrator terminal and a flame ionization detector. Samples were separated using a glass column (2 mm i.d. x 2 m) packed with preconditioned and pretested 10% Silar-10C on Gas-CHROM Q support (100-120 mesh, Applied Science, Inc., State College, Pennsylvania). This column was run at an initial temperature of 180 C for 10 min using nitrogen as the carrier gas at a flow rate of 25 ml/min. Thereafter, the temperature was increased at a rate of 5 C/min to a final of 225 C. The injection port and detector temperatures were set to 300 C, and the recorder was set at 100 mv full-scale deflection with a chart speed of 1 cm per min. Fatty acid methyl ester standards were obtained from Supelco, Inc. and Applied Science Inc.

Protein content was determined by the method of Lowry et al. (24).

RESULTS

Overall Lipid Analysis

The growth of *L. donovani* promastigotes in the macromolecule-free chemically defined RE-IX medium provided reliable quantitative measurement of a number of parameters. Total protein content of 10^9 cells was 2.8-3.0 mg, whereas total extractable lipids were 32.3% of total cellular protein and 12.2% of dry weight of cells. The contribution of isolated surface membranes and kinetoplast-mitochondria to total cellular protein and to total cell dry weight are given in Table 1. Further, the amount of total extractable lipids present in each of these subcellular fractions per mg protein and per dry weight of the fraction is also given in Table 1.

Total lipids of whole *L. donovani* promastigotes and their isolated SM and KM were fractionated according to their polarity using silicic acid column chromatography. The polar lipid fraction was found to be made up almost entirely of phospholipids. It comprised the major fraction and constituted almost the same percentage, 69-70%, of the total extractable lipids from whole cells, isolated SM and KM (Table 2). Phospholipids (PL) of the SM made up about 33% of their dry wt or 25% per mg of their protein, whereas the KM PL constituted ~17% of their dry weight and 19% per mg protein of these organelles (Table 2). The PL frac-

TABLE 1

Total Protein and Lipid Content of *L. donovani* Subcellular Fractions

Cellular fraction	% of whole cells		% lipid content	
	On protein basis	Dry wt basis	On protein basis	Dry wt. basis
Surface membranes	9.6	2.7	35.3	47.2
Kinetoplast-mitochondria	14.3	3.1	27.0	24.4
Whole cells	–	–	32.3	12.2

TABLE 2

Lipid Distribution in *L. donovani* Promastigotes: Whole Cells, Isolated Surface Membranes and Kinetoplast-mitochrondia

Cellular fraction/ Lipid fraction	Neutral lipids	Glycolipids	Polar lipids
Whole cells			
% of total lipids	24.1	7.2	68.7
% per mg protein	8.0	0.3	2.1
% of dry weight	2.9	0.9	8.3
Surface membranes			
% of total lipids	25.6	4.7	69.8
% per mg protein	9.1	1.7	24.7
% of dry weight	12.1	2.2	32.9
Kinetoplast-mitochondria			
% of total lipids	19.9	11.3	68.8
% per mg protein	5.6	3.2	19.3
% of dry weight	5.0	2.9	17.4

tion of the whole cells was only 2% and 8% per mg of cellular protein and dry weight, respectively. The glycolipid fraction was 5-11% of total extractable lipids in whole cells, isolated SM and KM, but it accounted for 1-3% when calculated on cell dry wt basis or 0.3-3% per mg of their protein. Neutral lipids, on the other hand, ranged between 20% and 25% of the total lipids of whole cells, SM and KM. This lipid fraction composed 12% and 9% of the SM, 3% and 8% of whole cells and 5% and 6% of the KM, calculated on a dry wt basis or per mg protein, respectively (Table 2).

Spent growth medium also was analyzed for possible released parasite lipids. After *L. donovani* cells were harvested from their growth medium as above, and the cell-free (0.45 μ filtered) spent medium was examined for lipid content by extraction and TLC (17,18, 25). No detectable lipids were found in such cell-free spent growth media.

Neutral Lipid Analysis

The neutral lipid fraction of whole cells and the 2 subcellular fractions were examined using silica gel HR TLC. Sterols were the predominant neutral lipid component (60%) in this fraction, followed by diglycerides (30%). Traces of monoglycerides, free fatty acids, sterol esters and other minor unidentified components also were present in this fraction. Further characterization of the neutral lipid fraction was not undertaken.

Glycolipid Analysis

Surface membrane glycolipid fractions were separated on Silica gel HR TLC plates (26). Upon charring or spraying with a phosphorus-detecting reagent, there were no detectable phospholipid or neutral lipid contaminants present in this fraction. However, 4 components were evident upon reaction with diphenylamine reagent. These glycolipids contained mannose- and galactose-moieties as determined using lectin binding (6). Further, these glycolipids had demonstrable antigenic activity as determined by agarose gel immuno-diffusion assays against rabbit anti-SM sera (6). Quantita-

TABLE 3

Phospholipid Constituents of *L. donovani* Promastigotes: Whole Cells, Surface Membranes and Kinetoplast-mitochondria

Component[a]	Lipid-P (%)[b]		
	Whole cells	Surface membranes	Kinetoplast-mitochondria
CL	1.9	0.6	10.5
PG	tra	tra	8.8
PE	13.3	37.7	10.7
PC	51.6	14.9	24.4
Unidentified[c]	18.5	19.0	27.5
PI	10.5	17.9	14.3
PS	1.0	9.9	3.9
PA	2.8	tra	tra

[a]PC = Phosphatidylcholine; PE = Phosphatidylethanolamine; PG = Phosphatidylglycerol; PS = Phosphatidylserine; PI = Phosphatidylinositol; CL = Cardiolipin, and PA = Phosphatidic acid.

[b]Lipid-P remaining at the origin was 3-5% of total and was subtracted.

[c]Using the TLC-solvent systems of Rouser et al. (17), this component had mean (n = 6) R_f values of 0.255 and 0.302 following separation in the first and second dimensions, respectively.

tion, specific identification and further immunochemical characterization of these 4 glycolipids currently is under investigation.

Phospholipid Analysis

The phospholipid fractions from whole cells, isolated SM and KM separated by silicic acid column chromatography were examined by 2-dimensional TLC. The identification and quantitation of these TLC separated components are given in Table 3. Qualitatively, the phospholipid profiles for whole cells, SM and KM are very similar. However, quantitatively, in whole cells, phosphatidylcholine (PC) composed more than 1/2 of the total phospholipids (PL), whereas phosphatidylethanolamine (PE) and phosphatidylinositol (PI) accounted for only 13% and 10%, respectively, of this fraction. Cardiolipin (CL), phosphatidic acid (PA) and phosphatidylserine (PS) constituted only very small portions of the total cellular PL. In contrast, the SM fraction had almost 38% of its total PL as PE, whereas PC constituted only 15%. The SM also contained elevated levels of PI (18%) and PS (10%), but only very small quantitites or traces of CL, PG or PA. On the other hand, the KM fraction contained 11% of the total PL as PE and 24% as PC and had elevated amounts of CL (10.5%), PG (9%) and PI (14%).

An unidentified phospholipid that had a mobility very similar to that of ceramide aminoethylphosphonate (27) also was present in considerable amounts in whole cells and the 2 subcellular fractions. Further, this unidentified compound constituted more than 1/4 of total PL of the KM fraction and almost 19% of both the SM and whole cell PL (Table 3). The chemical characterization of this as yet unidentified phospholipid has not been completed. However, it gave positive reactions for phosphorus, choline and vicinal hydroxyl groups but negative results for amino groups when sprayed with ninhydrin reagent (19). Acid hydrolysis and subsequent GLC analysis of this component (27) confirmed the presence of a sphingosine moiety. Complete characterization of this PL is currently under investigation.

Table 4 compares the diacyl, alk-1-enylacyl and alkylacyl contents of the major phospholipid components of the isolated SM with that of whole *L. donovani* promastigotes. The diacyl form was the predominant moiety of PC in both whole cells and the isolated SM. However, up to 1/3 of the PE content was present in the alk-1-enylacyl form in the SM but constituted only 1/5 of the PE in whole cells. The ether form of PI, on the other hand, constituted almost 1/4 of the total PI content of the isolated surface membrane PL but only 8% of the PI in whole cells. The diacyl form of PI constituted 60% and 84%, respectively, of the total PI content of isolated SM and of whole *L. donovani* promastigotes. Alkoxy-forms were not identified in any of the phospholipids isolated from either whole cells or their subcellular fractions.

Fatty Acids

The content and composition of various

TABLE 4

Diacyl, Alk-1-enylacyl and Alkylacyl Forms[a] of the Major Phospholipid Components of Whole *L. donovani* Promastigotes and Their Isolated Surface Membranes

	Surface membrane (% of total)			Whole cells (% of total)		
Phospholipid[b]	Diacyl	Alk-1-enyl-	Alkyl-	Diacyl	Alk-1-enyl-	Alkyl-
PC	92.6	7.4	trace	96	2	2
PE	43.1	33.1	23.8	68	20	12
PI	60.2	13.9	25.9	84	8	8

[a]Diacyl and alk-1-enylacyl forms were determined as described by Rinkonen (20) and Ferrell et al. (19), respectively. The dialkyl forms were determined by subtracting the sum of the content of diacyl and alk-1-enylacyl forms from the total.

[b]Abbreviations as in Table 3.

TABLE 5

Fatty Acid Composition of the Major Phospholipids of Isolated Surface Membranes and of Whole *L. donovani* Promastigotes[a,b]

	Surface membranes			Whole cells		
Fatty acid	PC	PE	PI	PC	PE	PI
<12:0	2.9	3.9	4.3	0.8	0.7	0.3
12:0	3.6	4.3	5.6	0.2	1.4	2.8
14:0	4.7	5.5	8.0	8.7	7.3	5.0
14:1	2.0	4.1	7.1	3.2	7.1	4.2
16:0	20.9	10.8	12.7	17.7	15.8	9.6
16:1	5.6	3.9	4.7	3.6	4.7	3.5
18:0	8.1	4.5	2.0	19.1	9.4	24.1
18:1	12.7	12.6	13.5	11.0	15.6	17.2
18:2	5.5	6.3	2.8	7.3	6.2	4.9
18:3	8.0	7.7	4.5	6.6	9.1	9.8
20:0	5.1	5.4	4.4	3.8	5.0	2.4
20:2	6.1	4.8	5.2	5.0	4.6	2.7
20:3	6.6	7.2	8.0	2.6	8.2	3.3
20:4	5.3	6.5	9.8	7.0	3.1	1.7
Unidentified	2.9	12.5	6.4	3.6	1.9	8.6

[a]Abbreviations for individual phospholipids as in Table 3.

[b]Fatty acid composition is given in mol %. Fatty acids are designated number of carbon atoms: number of double bands.

fatty acids esterified to the major phospholipids of isolated SM and of whole promastigotes are given in Table 5. The fatty acids 16:0, 18:1, 18:3 and the C20 series were esterified in similar quantities to PC of SM and of whole cells. However, C18:0 was the major fatty acid (19% of total) in PC of whole cells, whereas C18:0 constituted only 8% of the total fatty acid content of PC in the isolated SM. The mole ratio of unsaturated to saturated fatty acids in PC of SM was almost the same as that determined for whole cells (Table 6).

The major fatty acids esterified to PE of whole cells and of SM were C16:0 and C18:1 (Table 5). However, the fatty acids of PE in the SM contained 1/2 the amount of C18:0 and about 2/3 the amount of C16:0 of the PE in whole cells (Table 5). Moreover, the surface membrane PE contained greater quantities of unsaturated fatty acids in comparison to the PE of whole cells (Table 6).

The PI of whole cells showed pronounced differences in fatty acid composition in comparison to those of the isolated SM. While C18:0 and C18:1 made up about 24% and 17%, respectively, of the total fatty acids of PI in whole cells, they constituted only 2% and 14% of the total fatty acids of the surface membrane PI. Fatty acids of PI in whole cells also contained approximately 10% of C18:3 and 2% of C20:4. These 2 components constituted ~5% and 10% of the total fatty acids of the PI in the SM (Table 5). Moreover, the mole ratio of unsaturated to saturated fatty acids in the PI of

TABLE 6

The Mole Ratio of Unsaturated to Saturated Fatty Acids Esterified to Major Phospholipids of Whole Promastigotes and Their Isolated Surface Membranes

Phospholipid[a]	Surface membranes	Whole cells
PC	1.22	0.94
PE	2.59	1.51
PI	1.71	1.08

[a]Abbreviations as in Table 3.

SM and of whole cells was 1.7 and 1.1, respectively (Table 6).

DISCUSSION

A considerable body of data has accrued concerning the overall composition of *Leishmania* lipid classes and their metabolism. However, such lipid analyses have been confined to whole cells (28-33), causing much speculation as to the subcellular location of such *Leishmania* lipid components. The current study is the first to analyze the lipid content of surface membranes and kinetoplast-mitochondria isolated from *L. donovani* promastigotes and compare their content with that of intact cells.

The present results demonstrate that the surface membrane of *L. donovani* promastigotes possess a net enrichment in lipid content when compared to either whole cells or to the KM fraction. This is in general agreement with data for plasma membrane fractions from other protozoa and mammalian cells (34-36). This further confirms that the *L. donovani* SM preparation is a highly enriched subcellular fraction (6) and is characterized in this report by a low protein to high lipid ratio (i.e. greater than that observed for either whole cells or the KM fraction).

Currently PC, PE and PI were the major phospholipids identified in whole *L. donovani* promastigotes. These constituents are the same major phospholipids previously reported from whole *L. donovani* promastigotes (28,29). They also were observed in the same relative proportions as those reported for a number of trypanosomes and insect flagellates (36-41).

Analysis of the KM fraction indicated that PE, PC and PI were the major PL components. However, KM further contained remarkably high proportions of cardiolipin and PG in comparison to the isolated SM and whole cells. These results are not unexpected since high content of these 2 phospholipids is characteristic of mitochondrion lipids from mammalian cells (42). Although the *L. donovani* KM fraction contains $\sim$10-20% SM as a contaminant (6), the high levels of CL and PG are unmistakable characteristics of this subcellular fraction. Further, this subcellular fraction is enriched to $\sim \geq 80\%$ in kinetoplast-mitochondria as assessed by electron microscopy (unpublished observations).

The pronounced differences in the PL contents between whole cells, SM and KM infers a certain specificity as to the role of individual phospholipids in the physiological and biochemical functions of the latter 2 cellular compartments. It may be speculated that this specificity is requisite because, e.g., a membrane enzyme might have an absolute requirement for one or more specific phospholipid species to maintain its functional activity, or that the cell requires a precisely defined physical state (i.e. fluidity) within each membrane for it to achieve its physiological function. The exact reasons for such pronounced PL compartmental segregation obviously are not well established. However, CL was shown to restore activity to succinate cytochrome C reductase and cytochrome oxidase complexes in *Tetrahymena* (43,44) and to regulate ATPase activity in other eukaryotic inner mitochondrial membranes (45). Little information is available to suggest the role of CL in protozoan mitochondrial fractions, as most of the available information is derived from mammalian systems. However, by extrapolation, similar roles are, in all probability, applicable also to this unicellular system.

Phosphatidylcholine was present predominantly in the diacyl form in both the isolated SM and in whole *L. donovani* promastigotes. However, about 1/3 and 1/4 of the PE in the SM was present in the plasmalogen and ether forms, respectively. Further, approximately 1/3 of the surface membrane PI content also was present in the plasmalogen and ether forms. It is of importance to note that PE and PI together constitute >50% of the SM phospholipids and that ether linkages are not readily hydrolyzable by lipolytic enzymes. These observations suggest that the presence of these high levels of ether-linked lipids in the parasite surface membrane may afford it some protection against the hydrolytic enzymes present in both its insect and mammalian hosts.

The neutral lipid fraction of isolated SM accounted for about 20-25% of the total extractable lipids. Of this, sterols were the predominant constituents (60%). The currently observed sterol/phospholipid molar ratio is very similar to that previously reported for plasma membranes isolated from mammalian cells and other protozoa (35,36,41,46). The

high concentration of sterols in the SM of *L. donovani* might play an important role in the control of membrane fluidity as has been suggested for mammalian cells and for *Tetrahymena pyriformis* (46-49).

The major phospholipids (PC, PE and PI) of the SM and whole promastigotes contained C16:0, C18:0, C18:1, C18:2 and C18:3 as their major fatty acid constituents. More important, however, is the molar ratio of unsaturated to saturated fatty acids in these individual phospholipids. These unsaturated fatty acids may play an important role in the fluidity of the surface membrane which readily can be adapted to changes in environmental conditions (22,50).

The phase transition temperature of membrane lipids is highly dependent upon the length and degree of unsaturation of component fatty acids as well as the nature of the polar head group of the PL. These properties are profoundly affected by, and dependent upon, the environment in which the cell membrane exists. Therefore, the opportunities exist for metabolic regulation through surface membrane lipid compositional changes in *L. donovani* throughout its development in both its insect vector and mammalian hosts.

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Sterol and Fatty Acid Composition of the Clam, *Codakia orbicularis*, with Chemoautotrophic Symbionts

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ABSTRACT

Codakia orbicularis may obtain nutrients from chemoautolithotrophic bacteria. The chemical composition of the *C. orbicularis* was investigated because of this unusual source of nutrition, and because it is a human food source in the Caribbean. The lipid fraction of these molluscs is discussed in detail. Polyunsaturated fatty acids account for only 11-15% of the total fatty acids, and non-methylene interrupted dienes are present as high as 9.5%. Cholesterol represents about 45% of the total sterols present.

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INTRODUCTION

Bivalve molluscs traditionally have been thought to obtain nutrition solely from ingestion of photosynthetically-derived plant material. However, recent discoveries of large bivalves living in areas of hydrothermal activity in the deep sea (1-3) and the discovery of gutless bivalves (4,5) have necessitated a closer examination of bivalve nutrition. It now appears that animals within the families Lucinidae, Solemyacidae, Mytilidae and Vesicomyidae may obtain significant amounts of reduced carbon and nitrogen compounds from intracellular chemoautolithotrophic bacteria (6-15).

The Lucinid clams, especially *Codakia orbicularis* (Linné, 1758), are of additional interest, because they constitute an artesinal fishery for home consumption in the Caribbean (16). Unlike other clams with symbionts, this species is readily accessible in shallow waters and reaches a large size (98 mm maximum shell length). Our recent work on its morphology, nutrition and growth (14,15) showed that, in spite of its unusual energy source, *C. orbicularis* exhibits growth rates comparable to those of *Mercenaria mercenaria* (Gmelin, 1791), the common quahog of the eastern United States. This research investigates the chemical composition of the *C. orbicularis* meat in light of the clam's remarkable source of nutrition and limited capacity for sterol synthesis (17).

METHODS

Specimens of *Codakia orbicularis* were collected on two separate occasions, in the fall of 1981 and again in the fall of 1982, from shallow-water turtle grass, *Thalassia testudinum*, beds on the south shore of Grand Bahama Island, Bahamas (26°37'N x 78°23'W) and were brought to the laboratory in live, healthy condition. On each occasion, enough raw clams (about 50-60) were shucked and homogenized to yield a single 200 g composite sample. The shucking yield was about 22%. Aliquots were taken from this composite for the following procedures. Moisture content was determined by drying 10 g samples at 100 C to a constant weight. Ash content was determined on dried samples according to AOAC (18) procedure 18.025. Fat content was determined by the method of Bligh and Dyer (19).

A portion of the chloroform layer from the Bligh and Dyer extraction procedure was evaporated to dryness under nitrogen, saponified, and trans-esterified (20) for fatty acid analysis by gas-liquid chromatography (GLC). Fatty acid methyl esters (FAME) were separated on a Hewlett-Packard* (HP 5880) gas-liquid chromatograph (GLC) with a flame ionization detector (f.i.d.). Esters were separated on a 50 m x 0.20 mm i.d. wall-coated open-tubular (WCOT) fused silica column coated with Carbowax 20M. The carrier gas was helium at a split ratio of 100:1. The injector and detector temperatures were 275 C and 300 C, respectively. The oven temperature was held at 190 C for 27 min., long enough to allow elution of C18:0 and the C18:1 series. The oven temperature was then raised to 220 C at 1 C/min. and held at the final temperature for 26 min., long enough for C24:1 to elute.

Fatty acid methyl esters also were separated by thin-layer chromatography (TLC) on Silica Gel plates that had been soaked for 30 min. in 5% $AgNO_3$ in acetonitrile and activated at 100 C for 30 min. The esters were separated by

*Mention of a commercial company or product does not constitute an endorsement by NOAA, National Marine Fisheries Service.

degree of unsaturation using hexane:ether: acetic acid (90:10:1, v/v/v), scraped from the plates and prepared for GLC according to Dudley and Anderson (21). Samples were dissolved in hexane for injection into the GLC.

Identification was made by comparison of retention times of *C. orbicularis* FAME to those of available authentic standards, argentation thin-layer chromatography followed by GLC of the bands separated by degree of unsaturation, and GLC of the hydrogenated FAME. The thin-layer band containing the saturated fatty acid methyl esters also was analyzed by gas chromatography/mass spectrometry (GC/MS).

Polar and neutral lipids were fractionated by silica gel chromatography (22) using a portion of the chloroform layer from the Bligh and Dyer extract. Fatty acid methyl esters were prepared and analyzed as described above from both polar and neutral eluted fractions.

Sterol analyses were done in triplicate by direct saponification of about 2 g of the composite sample as described in the method by Kovacs et al. (23). The trimethylsilylether derivatives of the sterols were separated on a HP 5880 GLC with an f.i.d. A fused silica WCOT column (50 m × 0.20 mm i.d.) coated with SE-30 was used for the separation. Injector and detector temperatures were 250 C and 300 C, respectively. The column was held at 240 C for 10 min., then raised to a final temperature of 300 C at 3 C/min and held for 25 min. Quantitative analyses were done by adding 5-α-cholestane to each sample as an internal standard and running the samples against a standard cholesterol curve, plotting amount of cholesterol against peak area. For qualitative analyses, retention times and mass spectral data of the *C. orbicularis* sterols were compared with those of a standard containing brassicasterol, campesterol, stigmasterol and β-sitosterol (Supelco, Inc., Bellefonte, Pennsylvania).

RESULTS AND DISCUSSION

Values for fat, moisture, ash, and cholesterol are listed in Table 1. There was no significant difference between the proximate composition of the mollusc at the two different sampling times based on the sampling error of the methods used. The values, therefore, represent an average from the two sampling dates. Cholesterol was the major sterol, accounting for approximately 45% of the total sterols present. Other sterols identified were brassicasterol, campesterol, stigmasterol and β-sitosterol. Unidentified C_{26} and C_{27} sterols, with molecular ions at 370 and 384, respectively, accounted for 26.4% of the total (Table 2). The presence of plant sterols in bivalves has been well documented (24-28). Another sterol associated with bivalves and noticeable by its absence is 24-methylenecholesterol. Figure 1 shows the chromatographic trace of the sterols present in *C. orbicularis*. The total amount of cholesterol was very low at 21 mg/100 g meat, lower than any mollusc documented by Sidwell (29). The awning clam, *Solemya velum* (Say, 1822), also suspected of obtaining its nutrition from endosymbiotic chemoautotrophic bacteria (13, 30), is very unusual in having cholesterol constitute 100% of the sterol composition (31). Differences in sterol composition of bivalves may indicate major differences in metabolic pathways or composition of dietary sterols. Cholesterol dominates as the major sterol in hydrothermal vent clams, *Calyptogena magnifica*, and vestimentiferan worms, *Raftia pachyptila* (32). The vent waters and *R. pachyptila* contain the three major sterols, choles-

TABLE 1

Proximate Composition and Cholesterol Content for Raw *Codakia orbicularis*[a]

Analysis	Percent wet weight[b]
Fat	1.1 ± 0.1 (4)
Moisture	74.8 ± 1.5 (3)
Ash	2.9 ± 0.8 (3)
Cholesterol (mg/100 g)	20.6 ± 3.0 (6)

[a]See also ref. 14.

[b]± one std. dev. Number of samples is in parentheses.

TABLE 2

Sterols Present in *Codakia orbicularis*

Peak no. (Figure 1)	Sterol	RRT SE-30	Percent of total area
1	Cholestane	1.00	
2	C_{26} sterol	1.06	19.6
3	C_{27} sterol	1.85	6.8
4	Cholesterol	1.92	44.5
5	Brassicasterol	1.99	9.2
6	Campesterol	2.10	6.2
7	Stigmasterol	2.15	8.1
8	β-Sitosterol	2.26	5.5

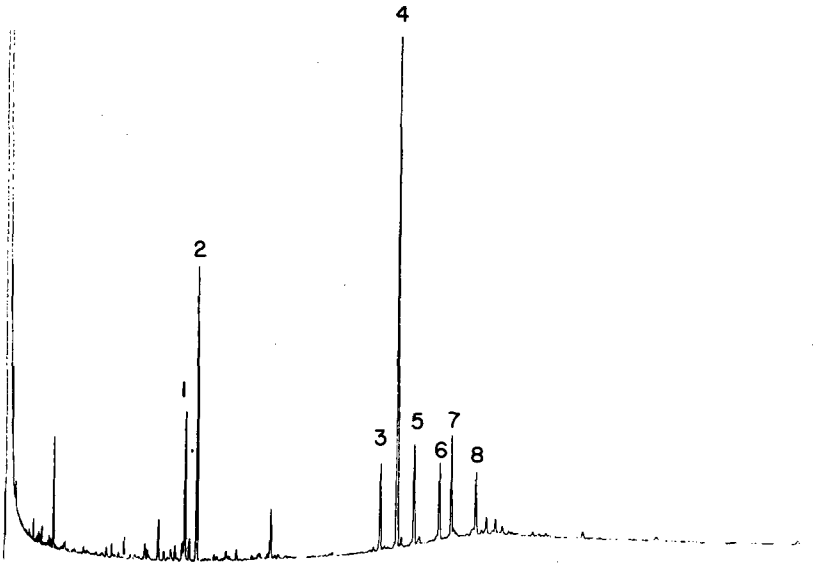

FIG. 1. Trimethylsilylether derivatives of *C. orbicularis* sterols separated by GLC on a WCOT fused silica column, 50 m × 0.20 mm, coated with SE-30. 1, cholestane; 2, C_{26} sterol; 3, C_{27} sterol; 4, cholesterol; 5, brassicasterol; 6, campesterol; 7, stigmasterol, and 8, β-sitosterol.

terol, desmosterol and 24-methylenecholesterol, but 24-methylene cholesterol is also absent from *C. magnifica* as was found with *C. orbicularis.*

Fatty acid compositional data are shown in Table 3. Only FAME commonly reported in the literature have been included in the table. Each value represents an average of two analyses obtained from two separate Bligh and Dyer extracts. No single fatty acid appears to be a prominent fatty acid, 18:1ω7 being present at only 12.5%. The lack of 22:6ω3 and the small amounts of polyunsaturates present are not surprising, because high levels of unsaturation probably are not necessary for survival of animals living in tropical waters.

The 18:1ω7 fatty acid is a precursor (33,34) in the biosynthesis of another prominent fatty acid, 22:2 (Δ7,15), a non-methylene-interrupted-diene (NMID) present at up to 9.5% of total FAME in *C. orbicularis* lipids. In fact, fatty acids having an ω7 olefinic bond, as indicated by the letter "b" in Table 3, account for a large percentage (about 30%) of the total fatty acids in this species, suggesting an important metabolic pathway. Only trace amounts of NMIDs were present in the neutral lipids; the greater portion appeared in the phospholipid fraction. Similar findings were reported (35) for *Mercenaria mercenaria* and speculation as to the physiological function of the NMIDs has been presented (36,37). Klingensmith (35) also found an inverse relationship between 22:2 NMID and the polyunsaturates 20:5ω3 and 22:6ω3.

Low amounts of plant-derived vitamins and other biochemical components in *C. orbicularis* (14) indicate that this species does not obtain significant nutrition from any of the available plant material. While 18:2ω6 and 20:4ω6 have been found in substantial amounts in marine algae, sea grasses and detritus (36), and are present at almost half the total polyunsaturates in the *C. orbicularis,* carbon and sulfur isotope ratios rule out the utilization of carbon derived from photosynthesis. Berg and Alatalo (14,15) have suggested that reduced carbon and nitrogen compounds in clam tissues are synthesized by intracellular symbiotic bacteria within the gills. Branched-chain fatty acids, commonly associated with bacteria, tentatively were identified in the *C. orbicularis* FAME. Three esters eluting between 16:0 and 18:0 were tentatively identified as *iso* and *anteiso* 17:0 and branched 18:0 in Table 3 based on the following: The ester labeled *anteiso* 17:0 eluted at the same retention time as the same standard compound; these esters migrated with the saturated FAME in argentation TLC; they had molecular ions at m/e 284 (for those identified as C17) and at m/e 298 (for those identified as C18), and their retention times did not change upon hydrogenation. These compounds were present collectively in the 1981 sample at 4.4% and in the

TABLE 3

Fatty Acid Composition of *C. orbicularis* Total Lipids

[a]Fatty acid methyl esters	Date of collection Fall, '81	Fall, '82
	Weight percent composition	
14:0	0.7	0.6
15:0	0.5	0.5
16:0	7.6	7.1
17:0	2.7	2.9
18:0	7.4	8.4
iso 17:0	1.5	0.8[e]
anteiso 17:0	1.4	0.5[e]
18:0 branched	1.5	1.1[e]
16:1 7	6.6	7.3[b]
18:1 9	2.6	1.9[c]
18:1 7	12.8	12.5[b]
20:1 11	5.1	6.0
20:1 9	1.7	1.3[c]
20:1 7	8.0	7.6[b]
18:2 6	2.4	3.0
18:3 3	2.5	3.0
20:4 6	4.9	7.2
20:5 3	1.2	1.6
20:2 NMID (Δ5,11)[d]	1.2	0.8[c]
20:2 NMID (Δ5,13)[d]	1.0	0.6[b]
22:2 NMID (Δ7,13)[d]	1.7	2.9[c]
22:2 NMID(Δ7,15)[d]	5.6	9.5[b]

[a]Shorthand notation for number of C atoms: number of double bonds and position of last double bond relative to terminal methyl group.

[b]Pathway to major NMID biosynthesis.

[c]Pathway to minor NMID biosynthesis.

[d]Non-methylene interrupted diene (Δ indicates speculative positions of double bonds relative to carboxyl end of the chain.)

[e]Tentative identification.

1982 sample at about 2.4%.

Two GLC peaks which are not included in Table 3, one eluting before 14:0 and one between 17:0 and 18:0, constituted about 3.5% of the total FAME and occurred only in the neutral fraction of the lipids. If the total saponified, methylated oil sample was first purified by TLC and the FAME band then chromatographed, these two compounds do not appear in the GLC trace. They also did not appear when the sample lipid was saponified and methylated in hexane and 2N methanolic KOH as opposed to MeOH-BF_3 methylation. This suggests that they are dimethyl acetal derivatives of plasmalogens (36). Glycerol ethers and plasmalogens have been reported in bivalves (37).

Codakia orbicularis appears to have similarities to other bivalves, such as the presence of plant sterols, non-methylene interrupted dienes and plasmalogens in the lipids. However, the small percentages of the polyunsaturates, 20:4ω6 and 20:5ω3, the absence of 22:6ω3, and the presence of branched chain fatty acids at 5% of the total FAME, combined with stable isotope and enzymatic data (14,15) indicate a bivalve mollusc that is metabolizing and/or feeding very differently from its conventional cousins.

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METHODS

Effect of Oxalic Acid Impregnation of Chromarods on the Separation of Phospholipids for Determination by the Iatroscan TLC/FID

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ABSTRACT

Phospholipid separations were carried out using Chromarods-SII impregnated with oxalic acid without interfering with Iatroscan TLC/FID detection and measurement. The resolutions were compared with untreated rods. Oxalic acid impregnated Chromarods gave better resolution of phospholipids, and the separations were more reproducible on a day to day basis compared to untreated Chromarods. A concentration of 0.25 M oxalic acid in acetonitrile, with 15 min of impregnation, followed by 60 min of activation at 110 C, provided the ideal conditions for coating. Three solvent mixtures, viz. $CHCl_3$:MeOH:HAc:H_2O (50:30:8:4, v/v/v/v), $CHCl_3$:MeOH:H_2O (65:35:4, v/v/v), and $CHCl_3$:MeOH:28% NH_4OH (70:30:2, v/v/v) were tested as developing solvents. $CHCl_3$:MeOH:H_2O (65:35:4) was found to be the best solvent system. Double development (initially in acetone, followed by $CHCl_3$:MeOH:H_2O [65:35:4]) is of minor value in improving separations. All the above solvent systems are capable of separating most of the commonly occurring plant phospholipids, except phosphatidylinositol and phosphatidylserine. Both of these phospholipids eluted together on Chromarods-SII, giving a single peak on the Chromarod, regardless of whether the rods were impregnated with oxalic acid.

Lipids 20:121-125, 1985.

INTRODUCTION

The use of the Iatroscan-TLC/FID for the measurement of phospholipids separated on Chromarods-S or -SII has been described by several workers (1). Tanaka et al. (2) originally investigated the reliability of Iatroscan in the determination of the composition of the polar lipids. The composition of cardiac phospholipids (3) and the separation of diacyl and plasmalogen phospholipids (4) have been examined using the Chromarod-Iatroscan system. However, there have been problems associated with the separation of certain types of phospholipids. Hiramatsu and Arimori (5), using $CHCl_3$:MeOH:H_2O (60:30:3.5) containing 500 mg/dl of butylated hydroxy toluene (BHT), found that phosphatidylinositol and phosphatidylserine always eluted together on Chromarods and gave a single peak on the Iatroscan. Vandamme et al. (6), using $CHCl_3$:MeOH:H_2O (80:35:3), separated lysophosphatidylcholine, sphingomeyelin, phosphatidylcholine and phosphatidylethanolamine, but were unable to separate cardiolipin, phosphatidylserine and phosphatidylinositol in the same phospholipid mixture. Innis and Clandinin (7) could separate the major phospholipid classes (diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylcholine, sphingomyelin and lysolecithin) by developing the Chromarods in light petroleum-diethyl ether (85:15) and $CHCl_3$:MeOH:H_2O (80:35:3) and specified the amount of water in the second developing system as a limiting factor for the separation. Instead of pure water, an oxalic acid solution (in water) has been used in the preparation of silica gel G plates for thin-layer chromatography (TLC) by Possmayer et al. (8) and by Iijima et al. (9) for the separation of phosphatidic acid from the total lipids, using petroleum ether (40-60°):acetone:formic acid (74:26:0.25) as the solvent system. We have now evaluated this modification of TLC techniques based on a silica gel Chromarod-SII impregnated with oxalic acid. It was found that improved resolution of phospholipids can be achieved with the modified Chromarods.

EXPERIMENTAL

Phospholipid standards (phosphatidylethanolamine, phosphatidylinositol, phosphatidylcholine, cardiolipin, sphingomyelin and phosphatidic acid) were obtained from Serdary Research Labs (London, Ontario, Canada). Phos-

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phatidylserine was obtained from Applied Science Labs (Pennsylvania, U.S.A.). According to the suppliers, the phospholipid standards were 96-99%; when the phospholipids standards were examined singly for purity, they produced 1 spot on the TLC plates and gave a single, symmetrical sharp peak on Iatroscan TLC/FID with the different developing systems described in the Results and Discussion Section. Oxalic acid (dihydrate) was Baker Chemical Co. (New Jersey, U.S.A.), ACS reagent grade. All organic solvents were redistilled under nitrogen before use.

The airflow on the Iatroscan TH-10 Analyzer (Iatron Labs, Japan, distributed in Canada by Technical Marketing Associates, world distributor Newman-Howells Assoc., Winchester, United Kingdom) was 2000 ml/min, the hydrogen pressure was 0.73 kg/cm^2 and the scan speed 2 mm/sec. Strip chart recorder Model 50 (Nissei Sangyo Instruments, Japan) was used with attenuation 10 mv and chart speed of 20 cm/min. Chromarods-SII (Iatron Labs, Japan), with a finer particle size than Chromarods-S, were used for all experiments.

The Chromarods were immersed in 30% nitric acid overnight, rinsed with distilled water and activated by passing through the FID of the Iatroscan immediately before use. The cleaned Chromarods were impregnated with oxalic acid by dipping the rods in a solution of the oxalic acid (in acetonitrile) for 15 min (or more in the case of optimization studies), activating them at 110 C for 30 min (or more in the case of optimization studies) and then spotting in the normal way. Standard mixtures of lipids in chloroform were applied (3 μl) by means of disposable pipettes ("Microcaps"–Drummond Scientific Co., Broomall, Pennsylvania) onto the rods. The Chromarods were kept in a saturated sodium chloride humidity tank for 10 min, developed for 40 min in the solvent system, dried at room temperature for 1 min, at 110 C for 2 min, and then scanned in the FID of the Iatroscan.

Standard solutions of samples were prepared by dissolving weighed amounts of standards in chloroform or chloroform:methanol (1 mg/ml). Each standard was stored under an atmosphere of nitrogen at −17 C.

Various concentrations of oxalic acid, different solvent systems and different experimental conditions were taken as parameters to optimize the best possible conditions. The details are given in the Results and Discussion Section.

RESULTS AND DISCUSSION

The decomposition of the formic acid molecule in the normal GLC type FID flame gives little or no signal (10,11). Oxalic acid is essentially 2 molecules of formic acid, and it has been shown that the GLC-FID response of dimethyl oxalate is essentially based solely on the 2 methoxy functions (12). This characteristic of oxalic acid suggested that this strong organic acid could be impregnated in the silica gel of the Chromarods-S or -SII to modify the chromatographic characteristics towards phospholipids without interfering with the Iatroscan FID performance. This has been found to be the case.

The humidity of the atmosphere, the surface condition of the Chromarod and the type of developing solvent system greatly influence the resolution of phospholipids, especially the separations among phosphatidic acid (PA), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) + phosphatidylserine (PS). On some days it was possible to obtain a resolution between PA, PE and PI + PS. However, the same rod, after cleaning and respotting with the same mixture of standard phospholipids, could then occasionally give a very poor separation of these phospholipids in which there was a partial separation between PA and the other phospholipids, with PE, PI and PS eluting together to give one peak. This indicates that the separations are not exactly reproducible on a day to day basis, for the same rod using the same solvent. However, much more reproducible separations could be obtained by impregnating the Chromarods with oxalic acid. The impregnated rods always showed a better resolution than the untreated ones, as illustrated in Figure 1, regardless of the other parameters, viz. concentration of oxalic acid, time of impregnation and developing solvent system. Of the different solvent systems tried, $CHCl_3$:MeOH:H_2O (65:35:4) was found to be the best for overall resolution and therefore, in one of the runs with this solvent system 2 more lipid standards, lysophosphatidylethanolamine (LPE) and lysophosphatidylcholine (LPC), were added to the "standard mixture of phospholipids" to investigate their behavior. The peak for LPE followed that of PI + PS and the peak for LPC that of PC. These two standards (LPE and LPC) were, however, not included in the "standard mixture" when other developing systems were tried.

With the impregnated rods a distinct separation was always obtained between PA and PE and also between PE and PI + PS. However, there was no separation between PI and PS (shown as PI + PS in figures), both being eluted as a single peak. Apart from the reproducibility of separation, the treated rods produce sharper

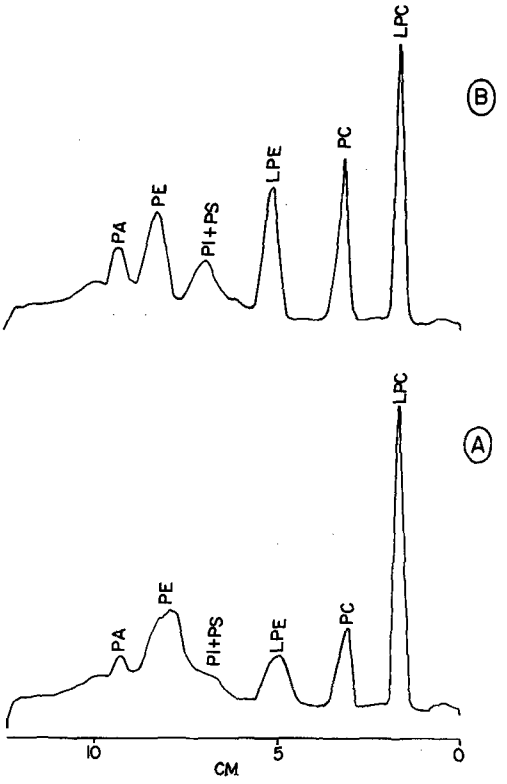

FIG. 1. Separation of phospholipids on Chromarods-SII. Development in: $CHCl_3$:MeOH:H_2O (65:35:4) for 40 min. (A) untreated; (B) oxalic acid impregnated.

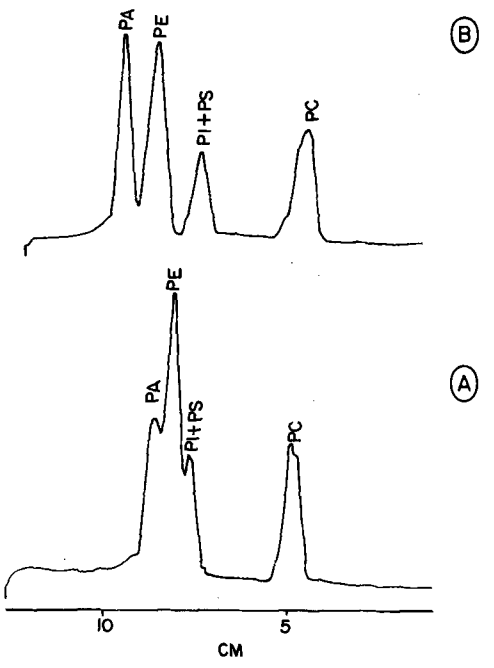

FIG. 2. Separation of phospholipids on Chromarods-SII. Development in: $CHCl_3$:MeOH:HAc:H_2O (50:30:8:4) for 40 min. (A) untreated; (B) oxalic acid impregnated.

and more symmetrical peaks than the untreated Chromarods-SII.

Several experiments were performed taking various concentrations (0.10, 0.15, 0.25, 0.30 and 0.35 M) of oxalic acid in acetonitrile for impregnation of Chromarods to find the most suitable concentration. Acetonitrile was preferred to water due to the ease with which it evaporates out from the surface of the Chromarods. The best concentration of oxalic acid for impregnation was found to be 0.25 M, regardless of the solvent system.

The Chromarods were immersed in 0.25 M oxalic acid for 15, 30 and 60 min, to investigate the effect of time of impregnation on the resolution of the various phospholipids. Longer impregnation did not have much effect, but 60 min of activation of the coated rods at 110 C, instead of 15 or 30 min, gave a better resolution of phospholipids. Possmayer et al. (8) and Iijima et al. (9) have used oxalic acid impregnated thin-layer chromatographic (TLC) plates for separation of phospholipids, particularly PA. The generally improved resolution of phospholipids on oxalic acid impregnated Chromarods observed in the present case probably is due to the formation of a complex between the inorganic phospholipid moiety and oxalic acid.

Developments of the impregnated Chromarods were tried in 3 different solvent systems, viz. $CHCl_3$:MeOH:HAc:H_2O (50:30:8:4, v/v/v/v), $CHCL_3$:MeOH:H_2O (65:35:4, v/v/v) and $CHCl_3$:MeOH:28% NH_4OH (70:30:2, v/v/v). Consistently, $CHCl_3$:MeOH:H_2O (65:35:4, v/v/v) was found to be the best solvent system for phospholipid separations on Chromarods-SII coated with 0.25 M oxalic acid. The elution order of the various phospholipids was similar when the rods were developed in $CHCl_3$:MeOH:HAc:H_2O (50:30:8:4) (Fig. 2A, without oxalic acid, Fig. 2B with oxalic acid) or in $CHCl_3$:MeOH:H_2O (65:35:4) (Figs. 1A and 1B). In both solvent systems PA was the most mobile phospholipid, followed by PE, PI + PS and then phosphatidylcholine (PC), but when $CHCl_3$:MeOH:28% NH_4OH (70:30:2) was employed as the solvent system, the elution pattern changed (Figs. 3A and 3B), the most mobile component being PE followed by PC, PI + PS and PA. This different resolution profile could be of importance for confirming the identity of individual phospholipid classes developed in the systems described above, through a different

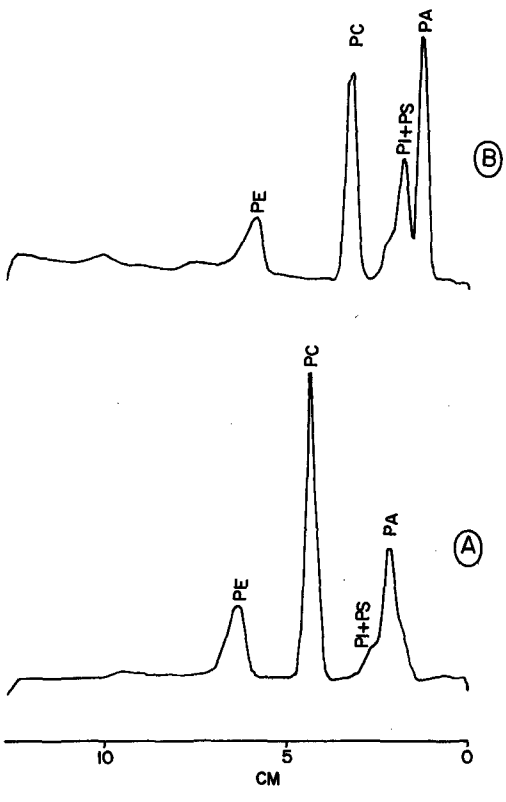

FIG. 3. Separation of phospholipids on Chromarods-SII. Development in: $CHCl_3$:MeOH:28% NH_4OH (70:30:2) for 40 min. (A) untreated; (B) oxalic acid impregnated.

elution profile. The oxalic acid had a relatively minor effect with the strong base in the solvent system (Figs. 3A and 3B).

A slight improvement of phospholipid separations also could be obtained by double development, i.e. initial development of coated rods in acetone, drying at room temperature for 2 min and a final development in $CHCl_3$:MeOH:H_2O (65:35:4). There was not much improvement to be expected when working only with polar lipids, since these are as such known to be not acetone-mobile (13). Very moderate shifts of components are, however, known to improve peak shape and response often (14). The effect of oxalic acid persists after the acetone development, suggesting that the oxalic acid is firmly bound to the silica gel, possibly as the anhydrous molecule. Oxalic acid loses water at 101-102 C.

In all the solvent systems tested, PI and PS emerged as one peak (PI + PS). This confirmed the findings of Vandamme et al. (6) who found, using $CHCl_3$:MeOH:H_2O (80:35:5), that there was no separation between PI and PS. Innis and Clandinin (7) suggested that the amount of water in the solvent system is very important in obtaining a clear resolution of these 2 phospholipids. However, various combinations of the volume ratios of $CHCl_3$:MeOH:H_2O did not bring any effective change in the resolution of PI and PS.

Sphingomyelin (SM) and cardiolipin (CL) are biochemically important phospholipids in many animal cells. Accordingly the behavior of these 2 additional polar lipids also was tested on oxalic acid impregnated as well as untreated Chromarods with development in $CHCl_3$:MeOH:H_2O (65:35:4). On both impregnated and untreated rods there was a good separation of SM from other phospholipids; it migrated in between PC and lysophosphatidylcholine (LPC). However, CL migrated along with PE and oxalic acid treatment of the Chromarod-S or -SII did not separate these 2 phospholipids with this solvent system. Recently Tanaka et al. (15) have obtained a baseline separation of CL and PE on Chromarods-A (alumina) instead of Chromarods-S, with development in $CHCl_3$:MeOH:H_2O:15N NH_4OH:pyridine (65:27.5:4:2:2).

One of the drawbacks of the oxalic acid impregnation, as with any other modified Chromarods, including addition of $AgNO_3$ (16), boric acid (17), or $CuSO_4$, is that the Chromarods need to be impregnated every time before use. Another limitation of this technique is that, if neutral lipids are separated first, followed by partial burn (19), the benefits of oxalic acid impregnation for polar lipid separations may be removed. Therefore, prior separation of phospholipids and neutral lipids by another chromatographic technique probably is required. A combination TLC/HPLC approach already has been proposed (20) as a solution to the crowding of polar lipids on HPLC, a somewhat similar situation.

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Mass Spectrometry of Isomeric Fatty Acid Hydroperoxides by Chemical Ionization via Direct Exposure Probe

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ABSTRACT

Intact isomeric methyl 9- and 13- hydroperoxy linoleates are analyzed by chemical ionization (CI) mass spectrometry using a direct exposure probe. Both isobutane and ammonia CI spectra were obtained. With isobutane CI, ions are observed for protonated fragments that are indicative of an acid catalysis mechanism similar to that observed in solution, whereas ammonia CI yields primarily adduct ions. The collisionally activated decomposition daughters of the high mass ions can be used to identify the position of the hydroperoxy group in the isomers studied.
Lipids 20:126-131, 1985.

INTRODUCTION

Isomeric unsaturated fatty acid hydroperoxides are important products in the oxidation of unsaturated fatty acids. Both the enzymatic and autoxidation of linoleic acid can result in mixtures of conjugated 9- and 13- hydroperoxy dienes. Additionally, small amounts of non-conjugated isomers (typically <1%) are found in autoxidation products (1).

Because unsaturated fatty acid hydroperoxides are thermally labile and thus unstable to analysis by gas chromatography-mass spectrometry (GC-MS), they customarily are converted to saturated methyl hydroxy fatty acids prior to analysis. The resulting hydroxy fatty acids then can be derivatized to trimethylsilyloxy groups and analyzed by GC-MS to determine the position of the hydroperoxy group along the fatty acid chain. Even though positional isomers of the trimethylsilyloxystearates are not completely resolved by GC, the composition of mixtures of simple allylic hydroperoxides can be determined by measuring the relative abundance of the scission products from the isomeric TMS derivatives and comparing them with standard mixtures (2).

An alternative procedure is the direct analysis of the intact hydroperoxides by MS. Electron ionization (EI) provides little, if any, useful information about the molecular weight for hydroperoxides because they are so labile. In a preliminary study, chemical ionization (CI) was shown to be useful for examining intact hydroperoxides, since intense high mass fragments indicative of molecular weight were observed (3). Fragments arising from CI of intact hydroperoxides can be isolated and studied by tandem mass spectrometry. In this report, we discuss the CI/MS spectra and MS/MS studies of intact isomeric methyl-9 and methyl-13-hydroperoxylinoleates, which were introduced into a triple quadrupole tandem mass spectrometer through a direct exposure probe.

EXPERIMENTAL

Methyl 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoate and . 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoate were prepared as described previously (4). Isomeric purity of the final products was >99% by HPLC (4). They were stored as dilute solutions (~0.5 mg/ml) in hexane at −20 C.

A Finnigan 4535/TSQ mass spectrometer was used in this study. This instrument consists of 2 independent quadrupole mass filters coupled in tandem and can be used for either normal MS scans or MS/MS experiments. Either isobutane or ammonia was used as the CI reagent gas at pressures of 0.1 to 0.5 torr as measured by the source pressure thermocouple gauge. The source temperature was maintained at 70 C unless otherwise noted. Samples were introduced via the direct exposure probe (DEP). One microliter of dilute solution of sample was deposited onto the tip of a rhenium sample filament. After solvent had evaporated by standing at room temperature, the probe was inserted into the MS. Under CI conditions, the filament is located approximately 1 mm from the exit aperture of the ion source and is completely surrounded by reagent gas plasma. The sample is then rapidly heated by programming the filament current to 400 ma at 20 ma/sec. The hydroperoxides eluted from the DEP as an approximately 3-sec-wide peak about 10 sec after heating was initiated. This corresponded to an estimated filament temperature of 200-250 C when the sample volatilized. The rapid heating of the probe gave a very rapid desorption profile of ions with a width similar

to a capillary GC peak profile. Intense spectra were provided by as little as 100 ng of hydroperoxide.

Data acquisition and mass spectrometer control were accomplished by the Finnigan data system. Software controlled both scanning and mass setting of both quadrupoles in MS/MS experiments. Conventional mass spectra were obtained by operating Q_1 and Q_2 in the all-pass modes while scanning Q_3.

Argon was the target gas in the collision cell (Q_2) for collisionally activated dissociation (CAD) experiments. The pressure of the collision cell was varied between 0.5 and 2.5 millitorr as measured by the Hastings gauge connected to Q_2. The collision energy for the CAD was controlled by the DC offset of the Q_2 rods. Typically it was at −20v. Daughter spectra produced in low-energy CAD experiments in quadrupole MS/MS are highly sensitive to both target gas pressure and collision energy. Careful attention to setting instrumental parameters is required to achieve reproducible daughter spectra.

RESULTS AND DISCUSSION

The CI (isobutane) spectra of the 9- and 13-hydroperoxy linoleate isomers are presented in Figure 1. The direct exposure CI technique yields spectra with more intense high mass fragments than reported previously (3) for a standard probe. Very little signal from the protonated molecule (m/z 327) is observed. A small signal at m/z 325 arises from the proton-abstracted molecule, and weak signals are observed from adduct ions of undetermined structure at m/z 349 and 351. Intense fragments at m/z 293 $(MH\text{-}H_2O_2)^+$, 309 $(MH\text{-}H_2O)^+$ and 311 $(MH\text{-}O)^+$ carry a large share of the total ion current.

As seen in Figure 1, additional fragments are observed in the spectra of the 2 hydroperoxide isomers. These fragments may arise by 2 mechanisms of decomposition: a homolytic decomposition mechanism and a heterolytic mechanism that appear to be analogous to the mechanisms of decomposition observed in the formation of volatiles from the hydroperoxides of methyl linoleate (4).

Fatty hydroperoxides decompose via β-scission of oxy radicals from homolysis of the hydroperoxide to form volatile compounds (5). Heating hydroperoxides in the injection port of a GC and separating the volatile products is a convenient way to initiate radical formation and separate the products formed. In addition, acid catalysis can lead to formation of a less complex mixture of volatiles by a decidedly different mechanism (4). Fragment ions in the

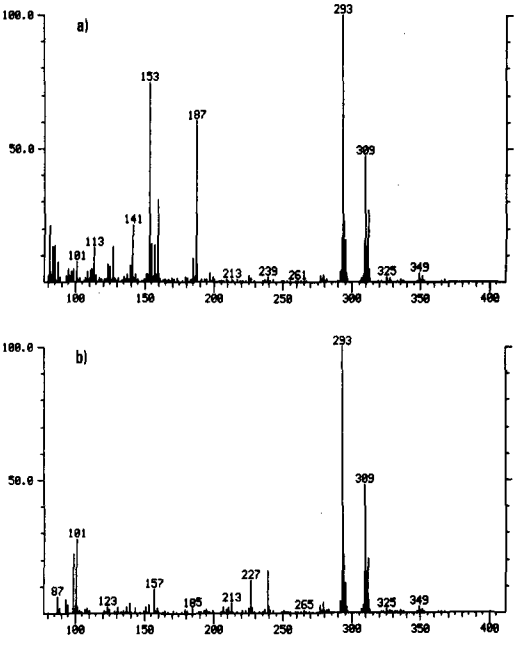
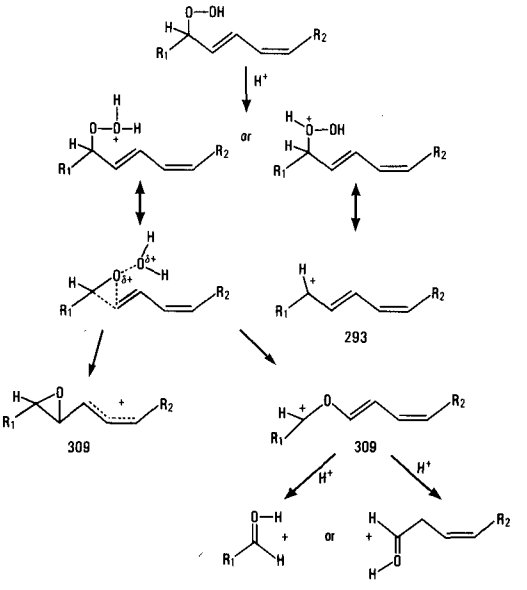
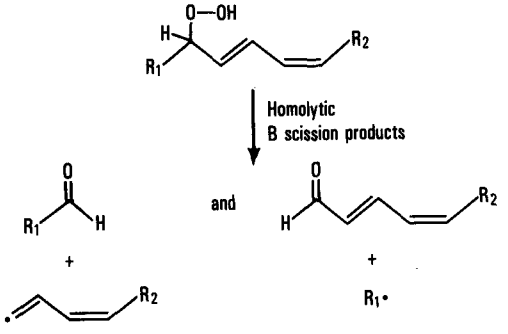


FIG. 1. Chemical ionization (isobutane) MS of (a) methyl 9-hydroperoxy-*trans*-10, *cis*-12-octadecadienoate, and (b) methyl 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoate.



SCHEME 1

isobutane CI spectrum of isomeric hydroperoxy linoleates can be accounted for with 2 fragmentation pathways: molecular protonation via isobutane, which leads to ions that can be compared with the acid-catalyzed fragmentation of linoleate hydroperoxides in BF_3-ether



SCHEME 2

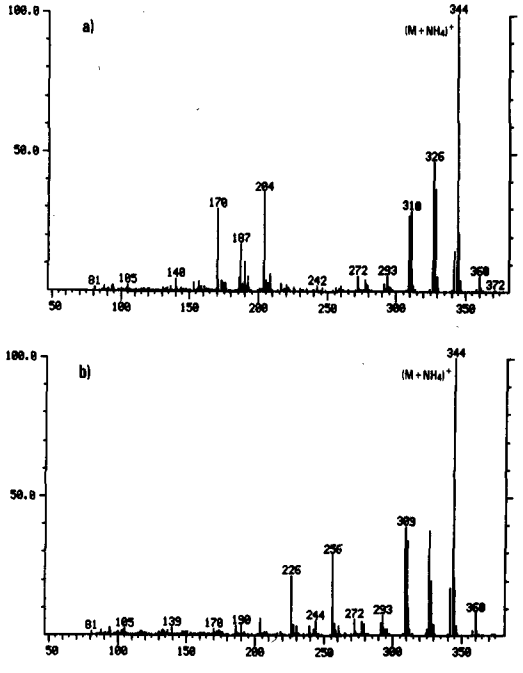


FIG. 2. Chemical ionization (ammonia) MS of (a) methyl 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoate, and (b) methyl 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoate.

(4) (Scheme 1), or oxy radical β-scission derived from homolytic cleavage of the hydroperoxide (Scheme 2). As discussed below, fragments indicative of both pathways are observed in the isobutane CI spectra of methyl 9- and 13-hydroperoxylinoleates. From the 9-hydroperoxide isomer, a fragment ion at m/z 187 (possibly protonated C9 aldehyde ester) could originate either by a heterolytic pathway or by β-scission of an oxy radical. Also, the m/z 101 ion (protonated C6 aldehyde) could arise from the 13-hydroperoxide by either pathway. More characteristic of only a heterolytic cleavage were an m/z 141 ion (protonated C12:1 aldehyde ester) from the 13-hydroperoxide. The fragment ions of m/z 159 (protonated C8 ester) and m/z 153 (protonated C10:2 aldehyde) from the 9-hydroperoxide were characteristic of the fragmentation expected from homolytic scission of an oxy radical. Likewise, a free radical cleavage of the 13-hydroperoxide may be the source of the m/z 239 ion (protonated C13:2 aldehyde ester). It is not completely clear whether these protonated fragments arise from decomposition of the molecule during evaporation from the probe followed bv protonation in the gas plasma or from fragmentation of the ions produced by reaction of the hydroperoxide with CI gas plasma ions.

With NH_3 as the CI gas, the adduct ion $(M+NH_4)^+$ becomes the base peak (Fig. 2). Ammonia CI is well suited for analyzing acid-labile compounds, such as hydroperoxides, since the $(M+NH_4)^+$ ion is more stable than the $(M+H)^+$ (6). Relatively less total fragmentation is observed in the ammonia CI spectra, although the fragmentation pattern is more complex. With the 9-hydroperoxy isomer, the C9 aldehyde ester and C10:2 aldehyde homolytic cleavage products are observed as their NH_4^+ adducts at m/z 204 and m/z 170. The C13:2 aldehyde ester from the 13-hydroperoxy isomer is observed as an NH_4^+ adduct by an intense peak at m/z 256. The heterolytic cleavage products from both isomers also are observed as their NH_4^+ adducts. Two additional signals are observed at m/z 140 in the 9-hydroperoxy isomer and m/z 226 in the 13-hydroperoxy isomer. It is not readily apparent whether these fragments are the unprotonated C9:1 and C12:1 aldehydes from heterolysis or whether they arise from a more complex route from an ammoniation reaction occurring in the CI plasma. Similar amines are reported to form from ketones in the CI source (7,8).

MS/MS experiments with CAD daughters of isomeric high mass fragments from hydroperoxides may prove useful in directly identifying and quantitating the hydroperoxide isomers in samples that have not been rigorously purified. These samples may contain other components with interfering ions at lower masses which complicate unambiguous identification of which hydroperoxide isomer is present based on the total spectrum. To examine this possibility, we have recorded CAD daughter spectra of the higher mass fragments that still could contain information describing the position of the hydroperoxy group on the fatty acid chain.

The 9- and 13-hydroperoxy isomers both produce intense ions at m/z 309 and 311 in the isobutane spectrum, as well as ions at m/z 309,

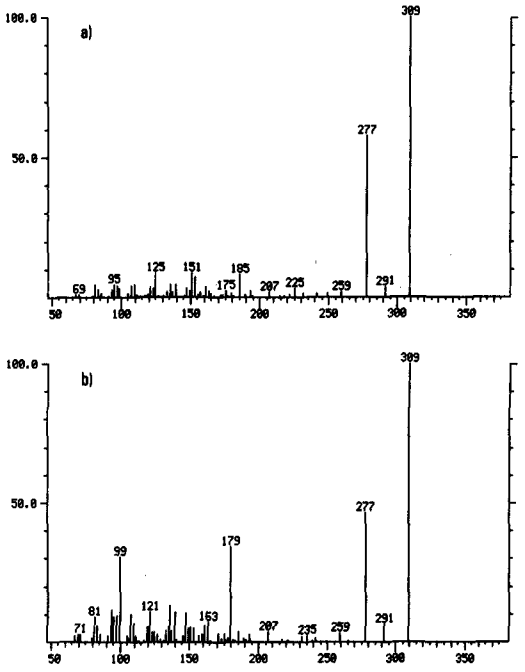


FIG. 3. Collisionally activated decomposition daughters of m/z 309 from (isobutane) chemical ionization of (a) methyl 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoate, and (b) methyl 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoate.

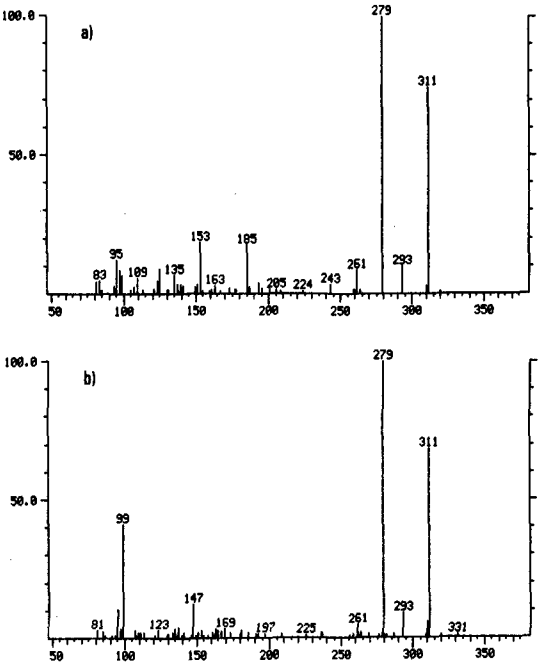


FIG. 4. Collisionally activated decomposition daughters of m/z 311 from (isobutane) chemical ionization of (a) methyl 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoate, and (b) methyl 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoate.

310, 326, 328 and 344 in the ammonia CI spectrum that are of different structure. All these ions contain at least one of the oxygen atoms from the hydroperoxide attached to the carbon backbone.

The CAD daughters of m/z 309 (isobutane) are clearly different from the 9- and 13-isomers (Fig. 3). The most intense daughter fragment from the isomeric m/z 309 parents arises from the loss of a neutral methanol molecule. It is observed at m/z 277. Additionally, the loss of water from the m/z 309 parent and the m/z 277 daughter are seen at m/z 291 and 259. These ions are not useful in locating the position of the oxygen on the parent sidechain. The relatively intense daughter at m/z 99 in the 13-hydroperoxy isomer and the m/z 185 daughter in the 9-hydroperoxy isomer can be explained with simple cleavages that do locate the original hydroperoxy group. These daughters arise from cleavage of the parent at C13 or C9 toward the saturated end of the molecule producing saturated carbonyl acyl ions of the type seen in EI spectra of saturated hydroxy fatty acids. The daughter spectrum of the m/z 307 ion in the CI mass spectrum (isobutane) of methyl-13-hydroperoxy linolenate shows a similar fragment at m/z 97 (data not shown). The intense daughter seen at m/z 179 in the 13-hydroperoxy linoleate isomer could be a cyclic rearrangement that has lost the C5 hydrocarbon tail (71 amu) and the carboxyl group (59 amu). A counterpart to this fragment is not seen in the 9-hydroperoxy isomers m/z 309 daughters. These daughter spectra are further complicated because the excess energy added to the parent by the CAD process causes isomerization of the double bonds and fragmentation which leads to a series of non-specific unsaturated hydrocarbon ions. The CAD spectra shown were taken at a Q_2 pressure of 1 mtorr with a collision energy of 20v. A large portion of the parent ion did not undergo decomposition. Further increases in either collision energy or pressure decrease the size of the parent peak in the daughter spectrum. However, the complexity of the fragmentation increases, but the abundance of the structurally significant daughters does not. At lower collision pressures and energies the parent and the fragment at m/z 277 arising from loss of methanol from the methyl ester become the only ions observed.

The CAD daughters of m/z 311 (isobutane) for both isomers also are unique (Fig. 4). The 9-hydroperoxy isomer gives strong fragments at m/z 153 and m/z 185 that are very small in the

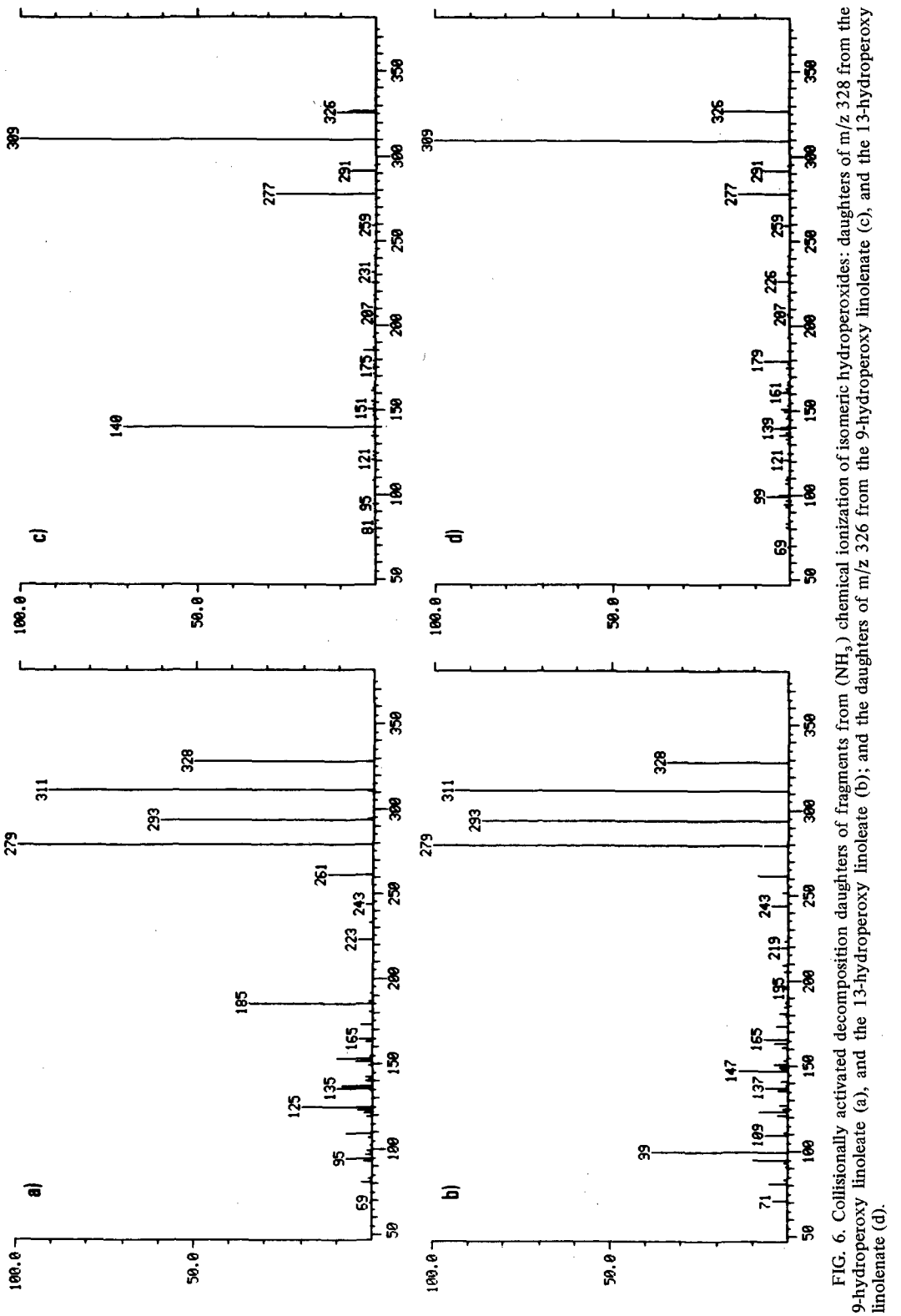


FIG. 6. Collisionally activated decomposition daughters of fragments from (NH_3) chemical ionization of isomeric hydroperoxides: daughters of m/z 328 from the 9-hydroperoxy linoleate (a), and the 13-hydroperoxy linoleate (b); and the daughters of m/z 326 from the 9-hydroperoxy linolenate (c), and the 13-hydroperoxy linolenate (d).

13-hydroperoxy isomer, whereas m/z 99 and 147 are intense in the 13-hydroperoxy isomer and small in the 9-hydroperoxy isomer. Again, the major daughter arises from loss of methanol from the parent.

The CAD daughters of the $(M+NH_4)^+$ adduct of the hydroperoxide isomers are complex spectra with many fragments (Fig. 5). The parent shows successive losses of NH_3, NH_3+H_2O, $NH_3+H_2O_2$ and $NH_3+H_2O+CH_3OH$ as intense ions. The fragment at m/z 225 is relatively more intense in the 9-isomer, whereas m/z 139 is larger in the 13-isomer; m/z 139 is an intense ion in both isomers, and most of the fragments observed are common to both isomers.

The CAD daughters of the m/z 328 (NH_3) and m/z 326 (NH_3) ions are the simplest and most strikingly different of all the parents (Fig. 6). The m/z 326 has the molecular mass of the parent hydroperoxide but probably is not a molecular ion. Rather, it either arises from loss of water from the adduct ion or the loss of water from an ammoniation reaction product in the gas plasma. Similar reactions are reported to occur (7,8) with ketones forming imines in the CI source. Of those ions tested for daughter spectra, those from m/z 328 probably are the best we have examined for giving a distinctly different spectrum for each of the hydroperoxide isomers. As seen in Figure 6, the intense ions at m/z 185 and 99 are particularly characteristic of the 9- and 13-hydroperoxides, respectively. These ions would appear to offer a good opportunity to analyze the isomeric composition of hydroperoxide mixtures. Other fragment ions also differ between the 2 isomers, such as m/z 125 and 153 for the 9-isomer and m/z 124 and 219 in the features of the 13-isomer.

The interesting gas phase chemistry that occurs in the CI source with NH_3 deserves much further attention with model compounds to unravel the details of just what structures are represented by the ions formed in NH_3 CI of substituted fatty acids. In any event, the technique of using MS/MS daughters should be helpful in studying these compounds. The unique daughters from m/z 309 and m/z 328 from the isomeric hydroperoxides may prove useful for quantitative determination of isomeric mixtures directly without reduction and hydrogenation.

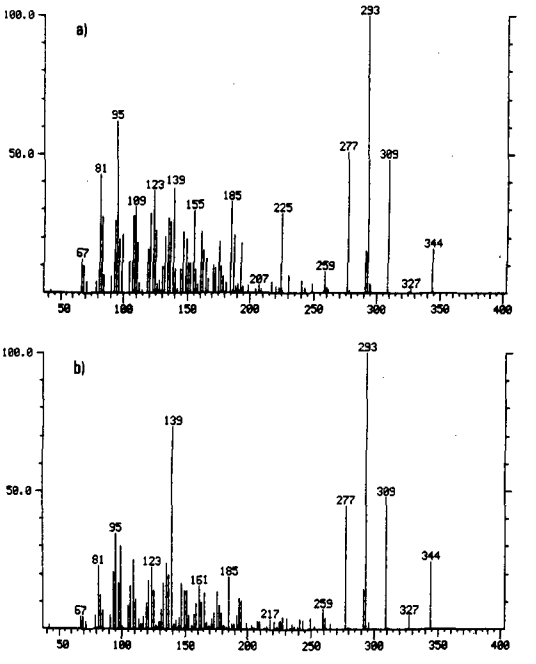


FIG. 5. Collisionally activated decompositions of the $(M+NH_4)^+$ adduct at m/z 344 from (NH_3) chemical ionization of (a) methyl 9-hydroperoxy-*trans*-10, *cis*-12-octadecadienoate, and (b) methyl 13-hydroperoxy-*cis*-9,*trans*-11-octadecadenoate.

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Metabolism of Lysophosphatidylcholine by Swine Platelets

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ABSTRACT

Incubation of intact platelets from Sinclair(S-1) miniature swine with ^{32}P-labeled lysophosphatidylcholine (lyso PC) indicated the presence of an active lysophospholipase with a pH optimum of 8.0 for hydrolysis of the substrate. However, lyso PC was incorporated into the membrane phosphatidylcholines by the acyltransferase pathway upon addition of ATP, Mg^{++} and CoA to the platelet suspension. These results suggest that intact platelets are able to resist the cytotoxic effects of lyso PC in plasma, and the phospholipids in platelet membranes are not readily affected by the lipid environment of the plasma. The acyltransfer reaction apparently is saturated with endogenous free fatty acids since arachidonic acid added exogenously did not further enhance the incorporation activity. Neither the acyltransferase nor the lysophospholipase activity was affected by Ca^{++}, but divalent metal ions such as Zn^{++} inhibited the lysophospholipase activity. Cholesterol but not cholesteryl esters elicited a biphasic effect on both enzymes, stimulating at low concentration but inhibiting at a cholesterol to lyso PC ratio greater than 1. Serum albumin inhibited the lysophospholipase but gave a small biphasic effect to the acyltransferase.

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INTRODUCTION

A considerable amount of lysophosphatidylcholine (lyso PC) is present in the plasma due to the action of lecithin:cholesterol acyltransferase (LCAT) (1,2). Because of their amphipathic properties, the lysophospholipids are cytotoxic and can cause lysis of cell membrane when present in excess (3). In the plasma, lyso PC tends to bind to lipoproteins with a much higher affinity than to albumin (4). Since there is no obviously active enzymic system in plasma to metabolize the lyso PC directly, the major route for its clearance is probably through uptake by various tissues and body organs (5,6). Within the cells, three major enzymic pathways have been identified for metabolism of lyso PC: (i) hydrolysis by lysophospholipases to form glycerophosphocholines (GPC) and free fatty acid (7); (ii) transacylation of two lyso PC molecules to form phosphatidylcholines (PC) (8), and (iii) acylation of lyso PC via the lysophospholipid:acyl-CoA acyltransferase to form PC (9). More recent studies have indicated other types of transacylases in which acyl groups are donated from PC to lysophospholipids such as lyso PE and lyso PS (10-12). These transacylase pathways are CoA-dependent and ATP-independent, and they are active in human platelets.

Since the blood cells are constantly embedded in an environment enriched in lyso PC, it would be important to understand the type of interaction between the cells and plasma with respect to metabolism of this lipid. Joist et al. (13) reported that lyso PC can cause both inhibition and potentiation effects on platelet functions, and these functional changes can occur at lyso PC concentrations which do not produce a gross structural modification or lysis of the membranes. In the past, studies with human platelets have presented evidence for degradation as well as conversion of lyso PC to PC (13,14). Intact human platelets were able to incorporate fatty acids into phospholipids, and this activity was enhanced by ATP, CoA and exogenous lyso PC (15). The acyl-CoA:1-acyl-GPC acyltransferase in platelet microsomes showed a high preference for acylation of arachidonic acid (16). The present study provides information regarding the metabolism of labeled lyso PC by intact platelets isolated from Sinclair(S-1) miniature swine. This animal model is a good source of intact platelets for biomedical research. The effects of divalent cations, albumin, cholesterol and cholesteryl esters on enzymes responsible for metabolism of the lyso PC are described. Successful studies using the swine platelets will be useful for future investigations correlating platelet membrane structure and functions.

MATERIALS AND METHODS

Materials

Sodium [^{32}P]phosphate (0.67 mCi/ml) was purchased from Mallinckrodt Diagnostics, St. Louis, Missouri. Other chemicals and reagents such as cholesterol, propranolol and phospho-

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lipase A_2 *(Naja naja)* were purchased from Sigma Chemical Co., St. Louis, Missouri.

Preparation of ^{32}P-lyso PC

Livers were taken from 16 rats that were injected i.p. 12-14 hr earlier with sodium [^{32}P]phosphate (0.27 mCi per 150 g rat). Lipids were extracted from the liver homogenate by chloroform/methanol (2:1, v/v), and ^{32}P-PC was isolated from the lipid extract by preparative TLC using chloroform/methanol/15N ammonium hydroxide (135:60:10, v/v/v) as developing solvent. The ^{32}P-PC recovered from the silica gel was further reacted with phospholipase A_2 (*Naja naja,* 100 μg/ml in 0.1 M Tris-HCl, pH 9) in an incubation mixture containing $CaCl_2$ and bovine serum albumin. After incubation at 37 C for 1 hr, 4 vol of chloroform/methanol (2:1, v/v) were added to terminate the reaction and extract the lipids.

The ^{32}P-lyso PC generated from the above procedure was isolated and purified on TLC plates with a solvent system containing chloroform/methanol/15N ammonium hydroxide (135:60:10, v/v/v). The purified ^{32}P-lyso PC was dissolved in a known volume of chloroform/methanol (9:1, v/v) and stored in the refrigerator until use. For determination of the specific radioactivity of the lyso PC, an aliquot was taken to measure the radioactivity and another for quantitation by GLC of the fatty acid methyl esters generated by base methanolysis with sodium methoxide.

Preparation of Platelet Suspension

For each experiment, 400 ml of blood were withdrawn from Sinclair(S-1) miniature swine, which are reared at the Sinclair Comparative Medicine Research Farm animal facilities. Blood was drawn by heart puncture directly into a 50 ml plastic syringe containing 10 ml of ACD (7.3 g citric acid, 22 g sodium citrate and 24.5 g dextrose/l distilled water) and then pooled into 150 ml siliconized glass centrifuge tubes containing 50 ml of 3% dextran in saline (138 mM NaCl, 5 mM KCl and 0.2% glucose). The samples were centrifuged at 110 g for 15 min at room temperature. The platelet-rich plasma was removed and centrifuged again at 150 g for 15 min to remove contaminating blood cells. The supernatant containing the platelets was centrifuged at 2500 g for 15 min. The pellet was suspended with 0.32 M sucrose in 50 mM Tris-HCl (pH 7.4) to make a final concentration of 2-8 × 10^9 platelets/ml. A 0.5 ml aliquot from the suspension was used for each incubation. Protein content of the suspension was also determined in some experiments by the method of Lowry et al. (17) using bovine serum albumin as standard.

Incubation of Platelets

All incubations were carried out in siliconized 10 ml glass tubes at 37 C in a shaking water bath for 30 min (unless otherwise stated). After ^{32}P-lyso PC was added and dried under N_2, buffer (0.32 M sucrose-50 mM Tris-HCl, pH 7.4) and co-factors (as required) were added. The reaction was initiated by adding 0.5 ml of platelet suspension (approximately 1 × 10^9 platelets) to make a final volume of 1 ml. After incubation, 4 vol of chloroform/methanol (2:1, v/v) were added to terminate the reaction. The reaction mixture was then separated into two phases by light centrifugation.

The lysophospholipase activity was determined by measuring an aliquot of the aqueous layer containing the radioactive GPC formed. Over 90% of the radioactivity of GPC was recovered in a single peak near the void volume after charging to a Dowex column. Acyltransferase and transacylase activities were determined by measuring the radioactivity of PC formed in the presence or absence of cofactors ATP (2.5 mM), Mg^{++} (5 mM) and CoA (0.1 mM). Tubes containing the same reaction mixtures but without incubation were used as blanks. To measure the PC formed, the organic phase was removed, and PC was isolated by TLC using chloroform/methanol/15N ammonium hydroxide (135:60:10, v/v/v) as solvent system. Radioactivity of GPC and PC was measured by a Beckman LS230 liquid scintillation spectrometer.

RESULTS

Incubation of platelet suspensions with ^{32}P-lyso PC resulted in a time-dependent breakdown of the substrate to form ^{32}P-GPC, which is recovered as a water soluble product. Under this condition, very little lyso PC was converted to PC, indicating that the lyso PC:lyso PC transacylase activity is very low. However, when ATP, Mg^{++} and CoA were added to the incubation mixture, a substantial amount of lyso PC was converted to PC (Table 1). The acyl transfer reaction apparently utilized endogenous free fatty acids, because addition of arachidonic acid (15 μM) to the incubation mixture did not further enhance acylation of ^{32}P-lyso PC to form PC. Results of this experiment also showed that activity of the lysophospholipase was not affected by the presence of cofactors for the acyl transfer reaction. Under the incubation condition in which co-

factors were present, Ca^{++} exerted little effect on the acyl transfer or the lysophospholipase activity.

Data in Figure 1 show the time-dependent utilization of ^{32}P-lyso PC by lysophospholipase and acyltransferase, i.e., incubation of lyso PC in the presence of cofactors. In general, the apparent lysophospholipase activity was considerably higher (8-12 nmole/30 min/mg) than that for the acyltransferase (1.4-4.3 nmole/30 min/mg). Activity of both enzymes also increased with respect to increasing amounts of protein, reaching a maximum around 0.7 mg for the acyltransferase and 0.9 mg for the lysophospholipase (Fig. 2). Lysophospholipase activity during incubation of 1×10^9 platelets (0.72 mg protein) increased linearly with substrate concentration up to 160 μM (Fig. 3). No inhibitory effect was observed even when the substrate was increased to 200 μM. When the assay was carried out in the presence of ATP and CoA, similar amounts of products were formed at all substrate concentrations tested, indicating that the two enzymes were not competing for the substrate. As shown in Figure 4, the lysophospholipase showed a pH optimum of 8.0, and little activity was found below pH 6.

Although neither enzyme activity was affected by Ca^{++}, other divalent metal ions such as Fe^{++}, Zn^{++} and Mn^{++} produced variable inhibitory effects on the lysophospholipase (Table 2). Among these, the inhibition by Zn^{++} was especially potent, giving rise to nearly complete inhibition at 3 mM. Surprisingly, cholesterol

TABLE 1

Effects of Cofactors on Activity of Lysophospholipase and Acyltransferase of Platelets from Sinclair (S-1) Miniature Swine

Cofactors	Enzyme products	
	PC	GPC
	cpm	
None	129 ± 24	3819 ± 66
ATP, Mg^{++}, CoA	1062 ± 92	3852 ± 42
ATP, Mg^{++}, CoA, 20:4	1012 ± 16	3732 ± 639
ATP, Mg^{++}, CoA, Ca^{++}	1036 ± 5	3826 ± 440

Platelets (1×10^9 cells in 0.5 ml) were incubated in a system containing ^{32}P-lyso PC (10,000 cpm in 100 nmoles) and 0.32 M sucrose-50 mM Tris (pH 7.4) in a final volume of 1 ml. Incubations were carried out at 37 C for 30 min. Concentrations of the cofactors were 2.5 mM ATP, 5 mM Mg^{++}, 0.1 mM CoA, 15 μM 20:4 and 3 mM Ca^{++} (with 1 mM EGTA). The activities of lysophospholipase and acyltransferase are indicated by the radioactivity of the enzyme products, GPC and PC, respectively. Data are the mean ± S.D. of 3 determinations and are representative of several experiments.

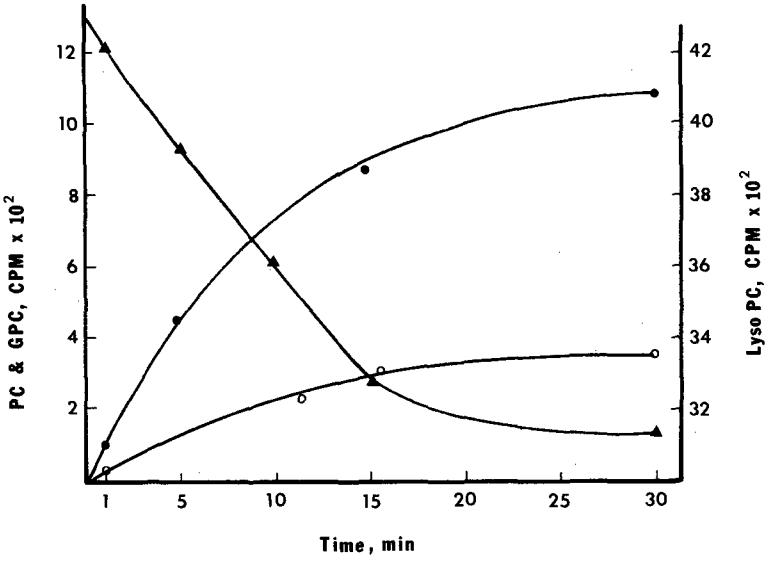

FIG. 1. Time dependence of PC and GPC formation and lyso PC degradation in platelets of Sinclair (S-1) miniature swine. Incubations were carried out at 37 C for 30 min in the presence of ^{32}P-lyso PC (10,000 cpm in 100 nmoles), ATP (2.5 mM), Mg^{++} (5 mM) and CoA (0.1 mM) and platelets (1×10^9 cells in 0.5 ml) in a total volume of 1 ml. ▲– – – – –▲, lyso PC; ●———●, GPC (lysophospholipase); ○———○, PC (acyltransferase).

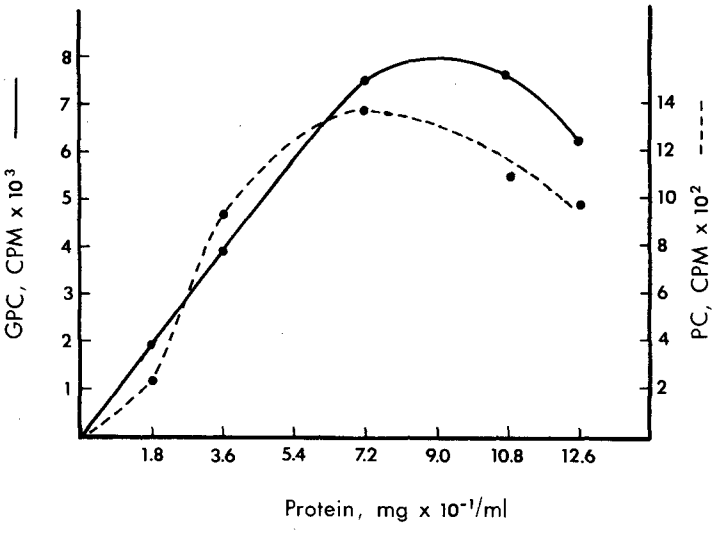

FIG. 2. Protein concentration curve for lysophospholipase (GPC) and acyltransferase (PC) in platelets of miniature swine. The condition for incubation was the same as in Figure 1, except that the protein level of the platelets was varied. The protein content of 1×10^9 platelets was 7.2 mg.

but not cholesteryl esters was shown to affect the lysophospholipid metabolism (cholesteryl ester data not shown). At a cholesterol/lyso PC molar ratio of 0.65, both lysophospholipase and acyltransferase activities in the platelets were stimulated, but as the ratio of cholesterol/lyso PC increased above 1, both activities were inhibited (Fig. 5). The inhibitory effect of cholesterol increased with increasing concentration, but no further inhibition was observed at concentrations higher than 200 nmole/ml. Bovine serum albumin also exerted an inhibitory effect on both enzymes, although the effect on lysophospholipase was more obvious (Fig. 6). Preliminary testing of the effect of propranolol (2.5 mM) indicated an inhibition to acyltransferase but not with the lysophospholipase (Table 2).

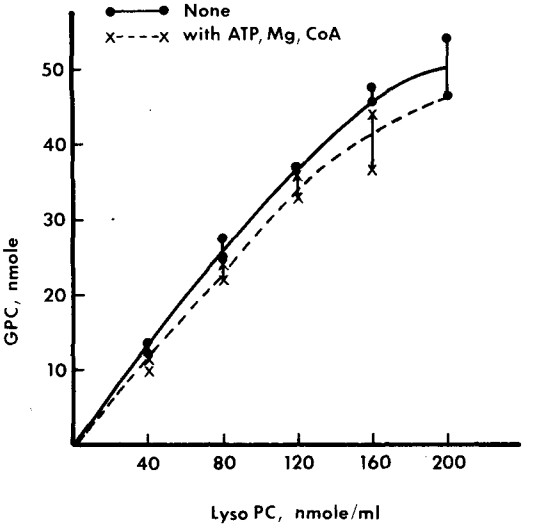

FIG. 3. Substrate concentration curve for lysophospholipase in platelets of miniature swine. Platelets (1×10^9 cells in 0.5 ml) were incubated at 37 C for 30 min in the presence of increasing concentrations of ^{32}P-lyso PC (nmoles/ml) either with or without cofactors for acyltransferase.

DISCUSSION

Results of the study indicated that when intact swine platelets were suspended in an incubation medium (pH 7.4) containing ^{32}P-lyso PC, the substrate was hydrolyzed mainly by the lysophospholipase to form GPC, but little PC was synthesized. This result seems to be different from those obtained by Elsbach et al. (14) with human platelets. However, the difference may be due to pH, because these investigators used pH 9 for the incubation. The fact that intact platelets can hydrolyze lyso PC added exogenously to the incubation medium implies that either the lysophospholipase is located at the external surface of the plasma membrane, or the substrate is readily diffused across the membrane for metabolism. Since platelet membranes had little ability to convert lyso PC to PC during incubation without added

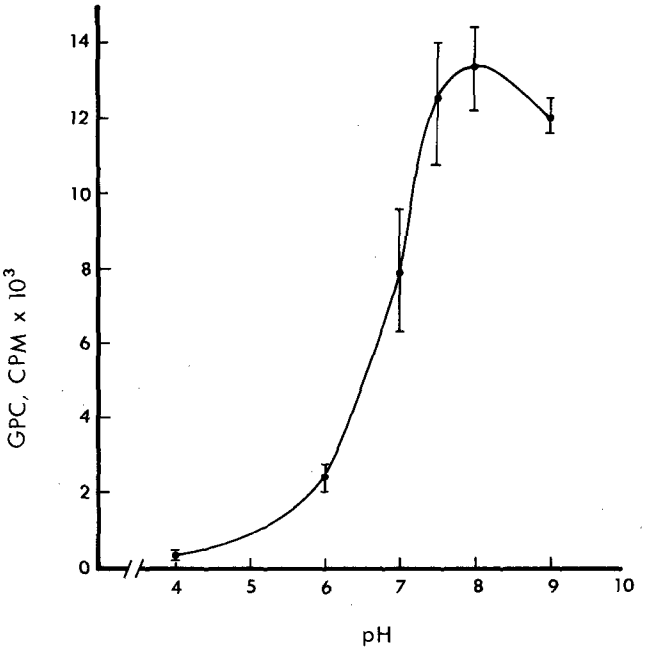

FIG. 4. Effect of pH on lysophospholipase activity in platelets of miniature swine. Platelets (1×10^9 cells in 0.5 ml) were incubated with ^{32}P-lyso PC in a system in which pH of the buffer was varied. Sodium acetate buffers were used for pH 4-7, and Tris-HCl buffers were used for pH 7-9.

TABLE 2

Effects of Divalent Metal Ions and Propranolol on Lysophospholipase and Acyltransferase of Platelets from Miniature Swine

Test compounds	Concentration	Lysophospholipase	Acyltransferase
		% of control	
Experiment A			
Fe^{++}	1.5 mM	93.0	–
Fe^{++}	3.0 mM	72.5	–
Zn^{++}	1.5 mM	26.5	–
Zn^{++}	3.0 mM	2.5	–
Mn^{++}	1.5 mM	62.0	–
Mn^{++}	3.0 mM	60.5	–
Experiment B			
Propranolol	2.5 mM	100	56.8

In experiment A, platelets (1×10^9 cells in 0.5 ml) were incubated with ^{32}P-lyso PC (100 nmoles) in the absence of cofactors. The reaction mixture contained 1 mM EDTA and various divalent cations to give a net concentration of 1.5 and 3.0 mM. Results are the average of triplicate incubations in which variances were less than 10% of the radioactivity measured. In experiment B, platelets (1×10^9 cells in 0.5 ml) were incubated with ^{32}P-lyso PC (100 nmoles) in the presence of cofactors as described in Table 1. Results are expressed as percent of controls which were incubated under the same condition, except in the absence of the inhibitors.

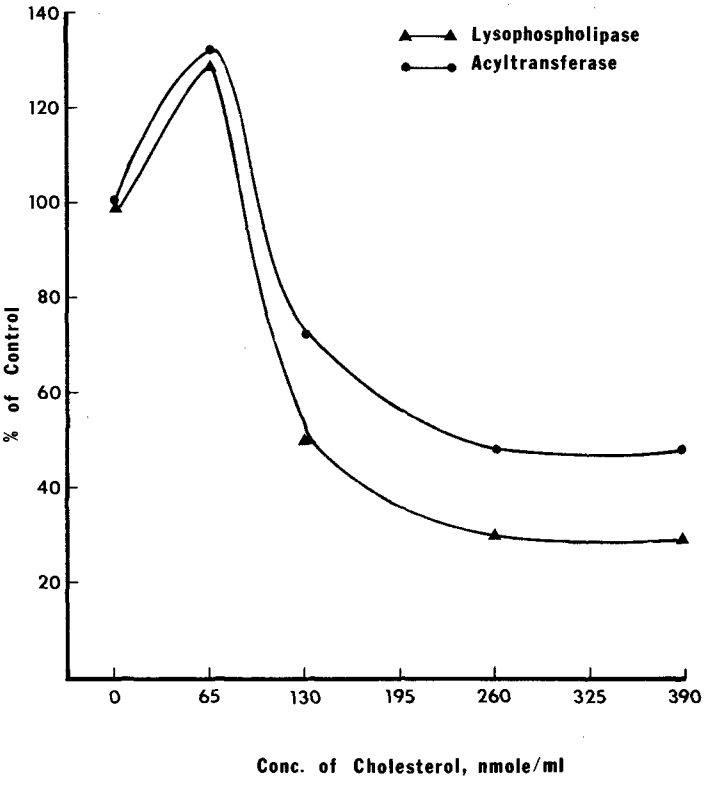

FIG. 5. Effect of cholesterol on lysophospholipase and acyltransferase activities in platelets of miniature swine. The condition for incubation of platelets in the presence of cofactors is described in Figure 1. The concentrations of cholesterol indicated do not include cholesterol present in the platelet membranes.

cofactors, the possibility of an active lyso PC metabolism via the lyso PC:lyso PC transacylase also was eliminated. Although Subbaiah and Bagdade (5,6) reported the presence of lyso PC:lyso PC acyltransferase in human plasma, this enzyme is found largely in the lung tissue for synthesis of lung surfactant (8). Nevertheless, our experimental results do not exclude the possible presence of other types of transacylases, especially those catalyzing the transfer of arachidonoyl-PC to lysophosphatidylethanolamine or lyso-phosphatidylserine (10-12). The lack of an active mechanism for incorporation of lyso PC from plasma into the platelet membrane phospholipids may be important in protecting against abrupt changes in lipid composition of the platelet membranes caused by short-term dietary influences.

Exactly why intact platelets require Mg^{++}, ATP and CoA for conversion of lyso PC to PC is not known. A similar finding was reported by Cohen et al. (15) for human platelets, although in their study labeled fatty acids were used. It is possible that the energy metabolites and acyl-CoA within the platelets were rapidly exhausted during isolation and centrifugation. Consequently, addition of the cofactors helped to restore the energy level required to convert the free fatty acids to their acyl-CoA and subsequently transfer the acyl-CoA to the lysophospholipids. This acylation pathway has been well-characterized by Lands and Crawford (9), and the enzyme(s) is present in most tissues and body organs. Surprisingly, addition of exogenous arachidonate did not further enhance the acylation activity, even though this fatty acid is regarded as the best acyl donor for the acyltransferase in many tissues (18,19). One explanation is that the membranes already are saturated with endogenous free fatty acids for the acylation reaction. Therefore, addition of exogenous fatty acids would not alter the rate of the acylation. Another explanation is that exogenously added fatty acids are not able to equilibrate effectively with the endogenous pool. The latter possibility actually has been demonstrated in other types of membrane studies in which free fatty acids were used as

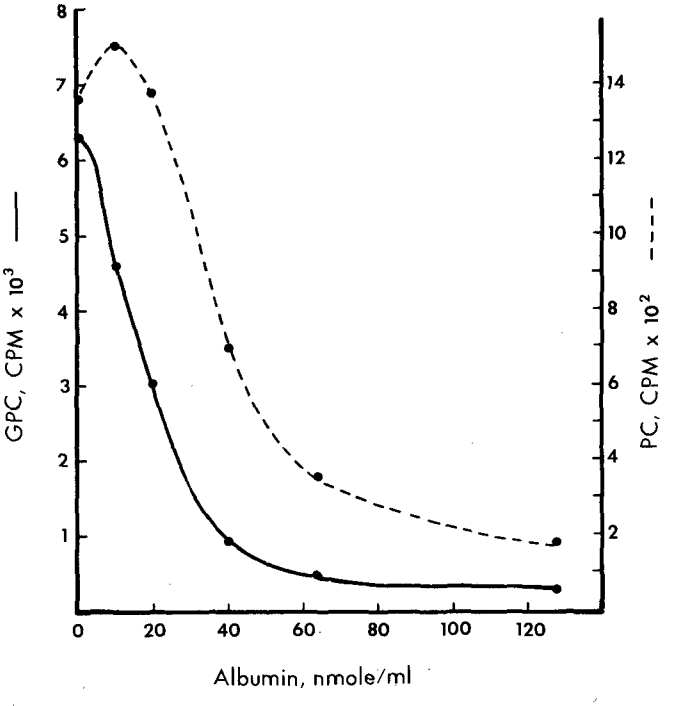

FIG. 6. Effect of bovine serum albumin on lysophospholipase (GPC) and acyltransferase (PC) activities in platelets of miniature swine. The activities were measured under standard assay condition as described in Figure 1.

substrate (20,21).

Although both lysophospholipase and acyltransferase can use ^{32}P-lyso PC as substrate effectively, the two enzymes do not seem to be competing for the substrate pool. The results further suggest that these two enzymes are operating at different sites in the platelet membranes, probably lysophospholipase at the outer surface of the membrane and the acyltransferase within the membrane matrix. Inhibition of the acyltransferase and lysophospholipase reactions was observed with respect to BSA, cholesterol but not with cholesteryl esters. The inhibitory effect exerted by cholesterol probably is due to formation of a tight complex between lyso PC and cholesterol (22,23). Apparently, the free hydroxyl group of cholesterol is important for the complex formation. Normally, there are more cholesteryl esters in plasma than free cholesterol, and both free and esterified cholesterols are bound to the lipoproteins. Lyso PC in plasma also is shown to bind to the lipoproteins (4), but to what extent individual lipid components of the lipoproteins may affect the platelet metabolism remains to be investigated. Since albumin cannot cross the membrane, it is expected that this protein would exert a more potent inhibitory effect on lysophospholipase than acyltransferase. Using this procedure, it is also possible to study effects of membrane active agents and drugs on platelet membrane enzymes. A preliminary testing with propranolol (2.5 mM) indicated that this compound exerted an inhibitory effect on the acyltransferase but not the lysophospholipase (Table 2). The specific action of propranolol, a cationic amphiphilic drug, further shows that the two enzymes are located at different sites of the membrane. Further studies with this drug are in progress.

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New Natural 2-Acetoxy Fatty Acids Using Chemical Ionization and Electron Impact Mass Spectrometry[1]

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ABSTRACT

The phospholipids of the sponge *Polymastia gleneni* contain saturated long chain (C_{22-30})-acetoxy fatty acids. Their structures were assigned based on chromatographic and spectrometric data as well as comparison with a synthetic sample. The use of capillary gas chromatography combined with chemical ionization and electron impact mass spectrometry was instrumental in the eludication of structures, since only a very small amount of crude lipids was available.
Lipids 20:141-144, 1985.

INTRODUCTION

The sponges are the most primitive multicellular animals. In the early fifties Bergmann and Swift (1) found very long chain fatty acids (17,20-hexacosadienoic and 9-hexacosenoic acids) in two marine sponges. More recently, additional new straight chain "demospongic" acids were reported by Litchfield and his coworkers (2-5). In our laboratory, nearly 50 novel acids were isolated from the phospholipids of various demosponges (6-12). All these phospholipid components have very long hydrocarbon chains ranging up to 30 carbons. In most cases, these acids contain the unusual $\Delta^{5,9}$-diene systems, but mono-unsaturation also is encountered (8,9,11). In addition, our recent studies revealed the presence of branched (6,7,11), methoxy (8,9), cyclopropane (10) or brominated (12) demospongic acids.

Sponges also are characterized by unusual ratios of phospholipid classes. In many cases larger amounts of phosphatidylethanolamine and phosphatidylserine rather than the "animal phospholipid" phosphatidylcholine are found. The unusual features of the phospholipids from such primitive organisms are of great interest in terms of biological membrane studies, an area which has drawn increasing attention in recent years. The occurrence of large quantities of PE and PS, together with the above mentioned unprecedented fatty acyl components, should have an effect on the physiological and morphological properties of the sponge cell membranes.

Our ongoing research of phospholipids from marine invertebrates has now uncovered another class of demospongic acids, namely totally saturated, 2-acetoxy C_{22-30} acids. These were isolated from a very small amount of a lipid extract derived from an extremely rare sponge, *Polymastia gleneni*, and their structures determined by means of capillary gas chromatography-chemical ionization and electron impact mass spectrometry.

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[1]Part 10 of "Phospholipids in Marine Organisms." For Part 9 in this series, see reference 12.

EXPERIMENTAL

Polymastia gleneni sponge colonies were collected in the Archipelago of Glenan, near Concarneau, France. They were extracted with cold $CHCl_3$/MeOH (1:1, v/v). The crude extract was purified by silicic acid column chromatography as described earlier (6). The extract was kept under argon or nitrogen at -10 C in solutions containing 0.002% BHT as preservative. Commercially prepared precoated silica gel TLC plates (0.2 mm thickness) were used for all TLC analyses. General reagents for phospholipid and fatty acid analysis were Molybdenum Blue and ceric sulfate. As a non-destructive spray reagent, Rhodamine 6G was used. Ninhydrin (primary and secondary amino groups, PE, PS), Dragendorff (tertiary and quaternary amino groups, PC) and Periodate-Schiff (carbohydrates, DPG, PG, PI) sprays also were used for the comparison of the phospholipid head groups with known standards.

A Carlo Erba series 4160 Fractovap chromatograph, equipped with a fused silica column (30 m x 0.32 mm) and coated with SE-54 (J & W Scientific, Inc.), a Model 400 LT programmer, a cooled on-column injection system and a flame ionization detector were used for the capillary gas chromatographic analyses. The initial and final oven temperatures were 130 C and 290 C for methyl esters and acetates, while the initial temperature was set at 190 C for the pyrrolidides. The temperature gradients were either 5 or 10 C/min. For the capillary gas chromatography-mass spectrometry measurements, a Ribermag GC-MS-DS system, combin-

ing a Ribermag R 10-10 quadrupole mass spectrometer with a Carlo Erba series 4160 Fractovap chromatograph, containing the same type of column used for the capillary GC analysis, was utilized. Optimum conditions in CI mode with ammonia were obtained by focusing on m/z 18 and m/z 35 with an average source temperature of 240 C.

Either a Varian Associates HA-100 or a Nicolet Magnetics Corporation 300 NMR instrument was used for 100 or 300 MHz NMR measurements. The solvent was deuterated chloroform. The values are given in ppm (δ). Infrared (IR) spectra were run using a Beckman Acculab spectrophotometer.

High performance liquid chromatography (HPLC) equipment included either a 50 cm x 9 mm Whatman ODS-2 reverse phase column or a 25 cm x 10 mm Altex Ultrasphere column, with a Waters M-6000A pump, a Valco loop injector and a Waters R401 refractometric detector. The eluting solvent was methanol.

The preparation of fatty acid methyl esters, N-acyl pyrrolidide derivatives and hydrogenation products was carried out as described earlier (6). Acetylation of hydroxyl groups was done using acetic-anhydride/pyridine (2:1, 24 hr) at room temperature. Standard fatty acid methyl ester mixtures needed for comparison purposes and for the determination of ECL values were obtained from Supelco (Supelco, Bellefonte, Pennsylvania) or from Applied Science (Milton Roy Co., Laboratory Group, State College, Pennsylvania). 2-Hydroxyhexacosanoic acid was purchased from Sigma Chemical Co., (St. Louis, Missouri).

RESULTS AND DISCUSSION

TLC analysis of the phospholipids indicated an unusual composition since some major spots furnished shorter Rf values than those of conventional phospholipids such as PE, PG, PC and PS. This suggested the presence of either substituted fatty acyl chains or new polar head groups, or both. The fatty acid methyl ester mixture obtained from the total phospholipids exhibited the presence of an additional spot with a lower Rf value (0.75 in hexane/ether [8:2, v/v]) than that of conventional fatty acid methyl esters, implying a substitution on the chain. The total fatty acid mixture was subjected to a small scale column chromatographic separation using silica gel as absorbent. Elution with hexane and hexane/ether (95:5, v/v) yielded the expected unsubstituted fatty acid methyl ester fractions. The major components in the mixture were 5,9-hexacosadienoic, 17-hexacosenoic and 5,9,19-octacosadienoic acids. $\Delta^{5,9,19}$-28:3 was the most abundant acid in the mixture. Smaller amounts of "conventional" fatty acids also were encountered. The use of hexane/ether (90:10, v/v) afforded the above mentioned polar TLC spot. Capillary GC analysis of this fraction showed the presence of five major and at least four minor compounds with unusually long retention times, which comprised approximately half of the total FAME mixture based on quantitative GC calculation. The increasing order in the ECL value of these compounds (24.39, 25.39, 26.39, etc., Table 1) implied a mixture of homologs. Hydrogenation of an aliquot of the mixture did not cause any change in the ECL values of any of the compounds, indicating a totally saturated composition.

An electron impact GC/MS analysis of the mixture gave essentially uninterpretable results. For example, the most abundant acid 3 furnished major fragment peaks at m/z 398, 381, 380, 366, 348 and 339. A fragment ion peak (McLafferty rearrangement) at m/z 74, typical for fatty acid methyl esters, was present but was very weak, suggesting a possible substitution on C-2.

An attempt to purify some individual acids from the above mentioned TLC spot did not

TABLE 1

2-Acetoxy Acids from the Phospholipids of *Polymastia gleneni*

Acid no.		ECL[a]	Percent
1	2-Acetoxydocosanoic (2-OAc-22:0)	24.39	13.4
2	2-Acetoxytricosanoic (2-OAc-23:0)	25.39	13.9
3	2-Acetoxytetracosanoic (2-OAc-24:0)	26.39	45.1
4	2-Acetoxypentacosanoic (2-Ac-25:0)	27.38	16.5
5	2-Acetoxyhexacosanoic (2-OAc-26:0)	28.38	7.5
6	2-Acetoxyheptacosanoic (2OAc-27:0)	29.38	0.6
7	2-Acetoxyoctacosanoic (2-OAc-28:0)	30.37	0.4
8	2-Acetoxynonacosanoic (2-OAc-29:0)	31.37	1.1
9	2-Acetoxytriacontanoic (2-OAc-30:0)	32.36	0.7

[a]Equivalent chain length values were calculated from the methyl esters of the corresponding acids.

provide sufficient material for spectral measurements. However, the NMR spectrum of the combined fractions displayed a terminal methyl triplet at 0.882 ppm and a large CH_2 absorbance at 1.259 ppm, in addition to two singlets (3H each) at 2.137 and 3.743 ppm. Methoxy carbonyl singlets arising from methyl esters of conventional fatty acids normally appear at 3.658 ppm. The down-field shift points to a possible α-substitution; for example 2-methoxy acids, obtained in this laboratory (8,9), give the methoxycarbonyl signal at 3.775 ppm, together with a methoxy singlet at 3.380 ppm. In the present instance, the presence of a singlet at 2.137 ppm raises the possibility of 2-acetoxy substitution. No absorption in the vinylic area was observed, confirming the previous hydrogenation results. The IR spectrum showed the expected hydrocarbon and ester bands at 3050 and 2820, respectively, as well as strong carbonyl absorption at 1740 cm^{-1}. An aliquot of the mixture was converted to pyrrolidides, but no further conclusive information was obtained from the mass spectra of these derivatives.

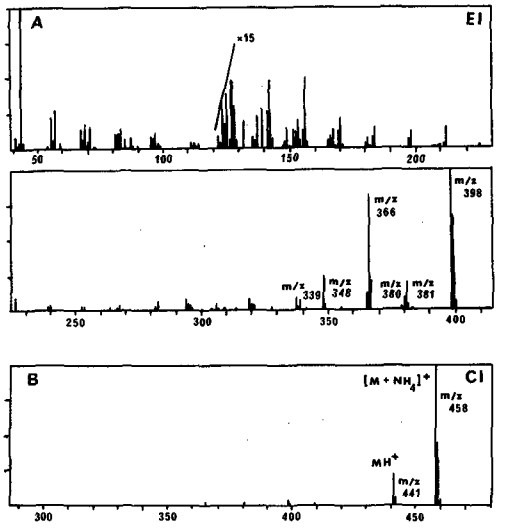

FIG. 1. Electron impact (EI) and chemical ionization (CI) mass spectra of 2-acetoxytetracosanoic acid (3, Table 1).

Since the above mentioned methods did not offer unambiguous and complete structures for our compounds, the mixture was subjected to further analysis using capillary gas chromatography-chemical ionization mass spectrometry with ammonia as the reagent gas. This time the molecular ions were clearly seen with intense M + 18 peaks, corresponding to ammonia adduct ions (Table 2). For comparison purposes, the electron impact and chemical ionization mass spectra of acids 3 and 5 are reproduced in Figures 1 and 2. The combination of data indicates a loss of a ketene group (M^+ - 42, m/z 398 in 3, and m/z 426 in 5, Figures 1-2), resulting from acetoxy substitution on C-2 of the fatty acids. The previously uninterpretable fragmentation ion peaks a, b, c, d, e and f now can be readily rationalized as shown in Table 2.

In order to confirm our assignment, commercially available 2-hydroxyhexacosanoic acid (2-OH-26:0) was subjected to methylation and acetylation in the usual manner. The resulting product gave the same TLC Rf value as well as NMR and IR absorptions reported for our natural mixture. In addition, capillary gas chromatographic, as well as EI and CI mass spectral properties of the synthetic compound and the natural acid 5 were identical in all respects. The

TABLE 2

Major Diagnostic Peaks in the Chemical Ionization and Electron Impact Mass Spectra of Acids 1-5 from *P. gleneni*

	Chem. ion.[a]		Electron impact[a,b]					
Acid	$[M^+ + NH_4]$	MH^+	a M^+-CH_2=C=O	b M^+-$COOCH_3$	c M^+-CH_3COOH	d M^+-(CH_2=C=O + CH_3OH)	e M^+-(CH_3COOH + CH_3OH)	f M^+-($COOCH_3$ + CH_2=C=O)
1	430	413	370	353	352	338	320	311
	(100)	(17)	(100)	(19)	(9)	(72)	(22)	(6)
2	444	427	384	367	366	352	334	325
	(100)	(24)	(100)	(21)	(12)	(85)	(24)	(9)
3	458	441	398	381	380	366	348	339
	(100)	(23)	(100)	(20)	(12)	(82)	(24)	(6)
4	472	455	412	395	394	380	362	353
	(100)	(18)	(100)	(11)	(6)	(44)	(13)	(4)
5	486	469	426	409	408	394	376	367
	(100)	(16)	(100)	(6)	(4)	(25)	(7)	(2)

[a]Intensities are given in parentheses.

[b]Peaks above m/z 150 are shown.

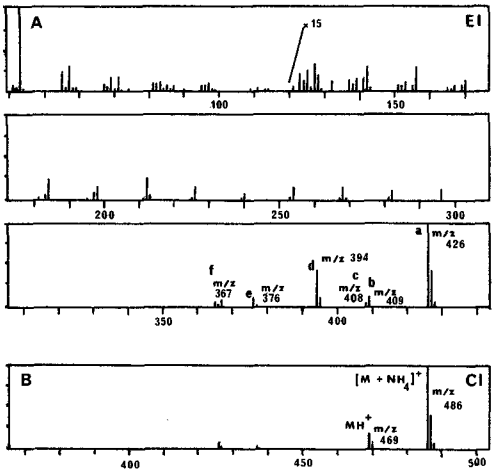

FIG. 2. Electron impact (EI) and chemical ionization (CI) mass spectra of 2-acetoxyhexacosanoic acid (5, Table 1).

above mentioned chemical and spectrometric data clearly establish the structures of the major compounds in the mixture as totally saturated 2-acetoxy C_{22-26} acids (Table 1). The minor components of our mixture, acids 6-9, which were obtained together with major compounds 1-5, did not give unambiguous mass spectra because of their very low concentrations. Based on the ECL values (Table 1) and biosynthetic considerations (6,7,9), their structures were assigned as 2-acetoxy C_{27-30} acids. Unfortunately, the small amount of material available prevented circular dichroism measurements of our acids in order to determine the absolute configuration of the acetoxy group. Naturally occurring 2-hydroxy acids are reported to have the *R* configuration (13). Our previous studies also indicated an *R* configuration for the 2-methoxy acids obtained from the phospholipids of the marine sponge *Higginsia tethyoides* (8,9). These findings lead us to suggest that the natural 2-acetoxy acids described in this paper also have the same configuration. To our knowledge, this is the first example of naturally occurring long chain 2-acetoxy acids in phospholipids.

The presence of substituted fatty acids with very long hydrocarbon chains as major components of total phospholipids of an organism is an unusual phenomenon in the biochemistry of cell membranes. Higher organisms are known to possess hydroxy fatty acids mostly in their nervous sphingomyelins. Our studies indicate the presence of methoxy, acetoxy or brominated acids in almost all classes of phospholipids. The necessity for such substitutions combined with very long hydrocarbon chains is still not known and calls for further investigation.

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Intestinal Cholesterol Uptake: Comparison Between Mixed Micelles Containing Lecithin or Lysolecithin

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ABSTRACT

The aim of our study was to define the mechanism by which cholesterol uptake is inhibited by lecithin but not by lysolecithin. The work compared the cholesterol uptake by everted rat jejunal sacs from bile salt-lecithin-cholesterol or bile salt-lysolecithin-cholesterol micelles. The micellar size and the cholesterol saturation were measured.

The size or molecular weight increases when the lecithin concentration rises, and the cholesterol uptake decreases and leads to zero when the micelles contain more than 30% lecithin. The size of bile salt-lysolecithin-cholesterol micelles is smaller than that of lecithin micelles in comparable molar ratios. Consistent with this result is the fact that, for a given phospholipid concentration, cholesterol uptake is greater in the presence of lysolecithin than in the presence of lecithin. The diffusion rate of the micelles through the unstirred water layer decreases when micellar size increases. However, the comparison of uptakes from lecithin or lysolecithin micelles similar in size and in cholesterol saturation showed that the cholesterol uptake is still lower for lecithin micelles. This shows that with larger micelles some factor other than micellar size and cholesterol content of the micelles is important. We observe that lysolecithin absorption is 15-fold greater than lecithin absorption. We suggest that lysolecithin absorption results in a rapid supersaturation with cholesterol leading to cholesterol absorption. *Lipids* 20:145-150, 1985.

INTRODUCTION

Several publications have demonstrated that the intestinal absorption of cholesterol and fatty acids from mixed micelles is partially inhibited by the presence of lecithins but not by that of lysolecithins (1-3). This inhibition seems to occur at the level of lipid uptake by the intestinal mucosa, but the mechanism remains to be elucidated. Two main hypotheses have been advanced as a function of current knowledge on lipid absorption. These are, first, that the rate of absorption depends on the velocity of micelle diffusion through the unstirred water layer. The inclusion of lecithins in the micelles increases micellar size and so decreases their rate of diffusion toward the epithelium (4,5). The importance of this diffusion velocity has been determined for other types of micelles (6,7). Second, the absorption of lipolysis products depends on their partition coefficient between the micellar phase and the juxta-membranous aqueous phase (8). This would explain why the absorption of cholesterol from mixed micelles of bile salts, fatty acids, monoolein and cholesterol depends on both the quantity of cholesterol present in the micelles and the degree of saturation of the micelles. Lecithins may increase the capacity of bile salt micelles to solubilize lipids, thus decreasing the release of monomers (9).

The role of these two parameters is not yet clearly established. The inhibitory effect of lecithins exists in conditions in which micellar size would not be excessively modified (4). In this case it is difficult to determine if the effect of lecithins on the solubilization of cholesterol can explain the results obtained, because mixtures with a highly variable composition have been used without determining the solubility limit of cholesterol in the mixtures.

In the present work we attempted to determine if the inhibitory effect of lecithin on intestinal cholesterol absorption could be explained by changes in micellar size and solubilization of cholesterol. This was done by in vitro comparisons of intestinal cholesterol uptake from mixed micelles of bile salt-lecithin-cholesterol and bile salt-lysolecithin-cholesterol, each with known size and degree of cholesterol saturation. The first step was the determination of conditions for optimal uptake of cholesterol from bile salt-lecithin-cholesterol micelles.

MATERIALS AND METHODS

Chemicals

Sodium taurocholate was purchased from Calbiochem, L α phosphatidylcholine (lecithin), L α lysophosphatidylcholine (lysolecithin) and

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cholesterol were 99% pure and were obtained from Sigma Chemical Co., St. Louis, Missouri. [4-^{14}C]-cholesterol (40-50 mCi · mmole^{-1}) and ^{3}H-cholesterol (50 Ci · mmole^{-1}) were purchased from CEA-France, and were found to be greater than 98-99% pure. [1-^{14}C] phosphatidylcholine and ^{3}H-inulin obtained from Amersham-France SA and [1-^{14}C] lysophosphatidylcholine from New England Nuclear, Boston, Massachusetts, were 97% and 98% pure. The radiochemical purity of the compounds was ascertained by thin layer chromatography (TLC).

Preparation of Mixed Micelles

The mixed micelles taurocholate-lecithin-cholesterol or taurocholate-lysolecithin-cholesterol were prepared by the coprecipitation method (10). The appropriate amounts of bile salt, lecithin, lysolecithin and cholesterol were dissolved in chloroform:methanol (2:1, v/v) in order to reach a final concentration of 10 mM bile salt with various lipid/bile salt ratios. The solvent was evaporated in vacuo over phosphorus pentoxide for 24 hrs, then the dried mixtures were dissolved in Krebs-Ringer bicarbonate (Ca^{++} omitted) pH 7.4. Micellar solubilities of cholesterol were determined as previously described (10) or from Carey's tables (11).

Molecular Weight Determination

Analytical ultracentrifugation was performed at 20 C in a Spinco-Beckman ultracentrifuge, model E, with speed and temperature controls. Sedimentation coefficient was measured using a double sector capillary cell with Schlieren optics. The Yphantis method (12) was used for the determination of micelle weights. The partial specific volume of mixed micelles was experimentally measured in a Parr microdensimeter (13).

Gel Filtration. The 8 mm x 100 mm glass column was packed with Ultrogel Aca 34 to a total bed volume of 37.7 ml. Eluted with 0.01 M Tris HCl, pH 7.5, 0.02% Na N_3, 8 mM Na taurocholate, blue dextran and vitamin B_{12} were used to determine the void volume (vo) and the total volume (vt).

The K_{AV} of the micellar elution volume was calculated as follows: $K_{AV} = (ve - vo)/(vt - vo)$ (14). This method gives an indirect estimation of the relative sizes of the different mixtures. The chemical determination of lecithin/bile salt/cholesterol peak obtained after chromatographic filtration showed the same molar ratio as the initial micellar solution. This method has been used for mixed micelles (4).

Cholesterol Uptake

The preparation of everted sacs has been described previously (6). Briefly, male Wistar rats (250-280 g) were fasted 12 hrs before experimentation and were killed by decapitation. The small intestine was removed and rinsed with cold saline and immediately everted over a glass rod. A 10-15 cm segment distal to the ligament of Treitz was used in this experiment. Sacs 1.5 cm long from this segment were tied off sequentially and kept in cold buffer solution until used. Incubation was made immediately at 37 C for 5 min and for a stirring rate of 750 rev/min^{-1} in micellar solutions which contained ^{14}C-cholesterol or ^{14}C-lecithin or ^{14}C-lysolecithin and trace amounts of ^{3}H-inulin as radiolabeled volume marker. Following incubation, sacs were removed, rinsed in cold saline and dried overnight at 60 C. Sacs were weighed and solubilized with Soluène-350 and Dimilume-30 (Packard Instrument, Downers Grove, Illinois) and the liquid scintillation counting was carried out using a TRI-CARB 300 C counter (Packard Instrument). The kinetic of cholesterol uptake has been measured between 3 and 15 min. A linear relationship exists between the amount of cholesterol uptake and time. Therefore, a 5-min incubation time was chosen because 5 min are sufficient for the unstirred water layer to become uniformly labeled with the nonpermeant marker and not too long to damage the membrane. The data were expressed as nmol of cholesterol, lecithin or lysolecithin per 100 mg tissue dry weight and per 5 min incubation. The results were given as means ± SE and were compared by using Student's t-test.

RESULTS AND DISCUSSION

Effect of Lecithin Concentration on Cholesterol Uptake

The different taurocholate-lecithin-cholesterol mixtures were all saturated with cholesterol. Thus, the cholesterol concentration varied in each mixture and increased in parallel with that of the lecithins. It is known that uptake depends not only on the degree of saturation of the micelles, but also on the cholesterol concentration in the mixtures. In these conditions, we should observe an uptake which is proportional to the quantity of cholesterol and thus to lecithins present. The results in Table 1, however, show that the actual situation is more complex. Cholesterol uptake increases with lecithin concentration only when lecithin concentration is lower than 2.1 mM. This increase seems to be related to the low concentration of cholesterol solubilized in micelles

TABLE 1

Effect of Varying Concentrations of Lecithin on Cholesterol Uptake

Concentration in micellar mixtures				
Lecithin (mM)	Cholesterol (mM)	n	Cholesterol uptake (nmoles·100mg^{-1}·5min^{-1})	Apparent adherent fluid volume (μl·100mg^{-1}·5min^{-1})
1.20	0.21	12	8.40 ± 1.02	43.83 ± 2.86
2.10	0.38	12	17.65 ± 0.80	54.30 ± 2.33
2.42	0.45	15	12.40 ± 2.15	57.79 ± 2.34
3.00	0.57	21	5.53 ± 0.82	60.81 ± 5.09
4.40	0.75	12	0.91 ± 0.52	62.55 ± 3.55
6.00	0.75	12	0	54.15 ± 2.16

The taurocholate concentration is kept constant (10 mM), and cholesterol concentrations used correspond to maximal cholesterol solubilities determined by Carey's table.
Values are mean ± SE. n = number of animals.

containing less than 2.1 mM lecithins. On the contrary, when lecithin concentration increases from 2.1 mM to 6.6 mM, cholesterol uptake decreases in spite of the increasing amount of cholesterol solubilized by the micelles. Cholesterol uptake is completely abolished for lecithin concentration greater than 4.4 mM. The decrease of cholesterol uptake seems well explained by changes in micellar size. The results in Table 2 clearly show that, when lecithin varies from 2.1 mM to 6.0 mM, the size of the taurocholate-lecithin-cholesterol micelles doubles. The apparent molecular weight increases from 50,000 for 1.2 mM lecithin concentration to 124,000 for 6 mM lecithin concentration in mixed micelles. The results obtained by gel filtration method (K_{AV}) confirmed the changes of micellar size observed by analytical ultracentrifugation. It is to be noted that the methods used furnish only an apparent mean molecular weight and do not account for eventual structural changes as a function of concentration or polydispersity. Prior work has shown that this type of change is not extensive in our conditions (15). The values obtained after ultracentrifugation indicate that the molecular weight of the mixed micelles is slightly higher than published values (16). The different results are difficult to compare, because the experimental conditions are not exactly the same. It should be noted that our results obtained with ultracentrifugation and gel filtration are in entire agreement in terms of the micellar size change as a function of lecithin concentration. In addition, the migration buffer for ultracentrifugation or gel filtration included 8 mM taurocholate, reducing the risks of micellar rearrangement. For polydispersity it appears from the data of Mazer et al. (17) that in the concentration range we studied with lecithin two micellar species coexist, simple bile salt micelles and mixed micelles. Simple bile salt micelles have a negligible solubilization power for cholesterol and do not interfere directly with cholesterol uptake. Mixed micelles which contain the cholesterol have, for a low lecithin concentration, a very low index of polydispersity (20%) and the mean molecular weight must be meaningful. The overall result is consistent with a major influence of micellar size (above 63,000) on cholesterol uptake for a lecithin range between 2.1 mM and 4.4 mM. On the contrary, below this value the effect of micellar size becomes negligible in contrast to the effect of the quantity of cholesterol solubilized which becomes predominant. The result obtained appears to be explicable without incurring a specific effect of lecithins except for modifications of micellar structure, even though such an effect cannot be excluded (18).

In order to more precisely examine the possible existence of specific inhibition of choles-

TABLE 2

Effect of Varying Concentrations of Lecithin on Apparent Molecular Weight of Mixed Micelles Taurocholate-Lecithin-Cholesterol

Concentration lecithin (mM)	Ultracentrifugation			Gel filtration
	V[a]	S[b]	aMW[c] daltons	KAV[d]
1.20	0.928	1.91	50,000	–
2.10	0.927	1.98	63,000	0.75
2.42	–	–	–	0.70
3.00	0.927	2.24	73,000	0.60
4.40	–	–	–	0.52
6.00	0.924	2.54	124,000	0.37

[a]Partial specific volume.
[b]Sedimentation coefficient.
[c]Apparent molecule weight.
[d]KAV = (elution volume - void volume) (total volume - void volume).

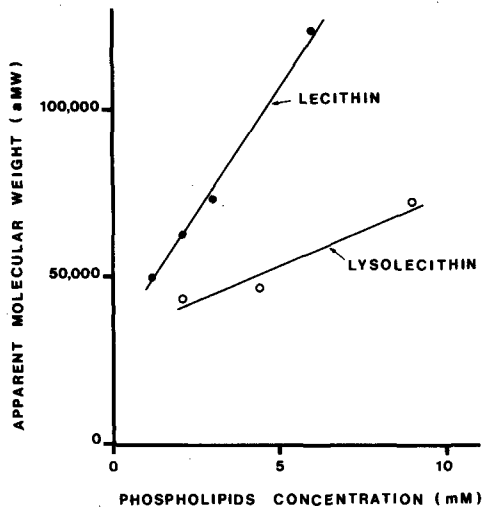

FIG. 1. Apparent molecular weight of mixed bile salt-lysolecithin-micelles versus mixed bile salt-lecithin micelles is determined by analytical ultracentrifugation. The bile salt concentration is 10 mM and mixed micelles are saturated with cholesterol.

terol uptake, we investigated cholesterol uptake from micellar bile salt-lysolecithin-cholesterol mixtures.

Cholesterol Uptake from Bile Salt-Lysolecithin-Cholesterol Mixtures

Before studying cholesterol uptake it was necessary to determine the cholesterol solubilizing capacity and micellar size of bile salt-lysolecithin mixed micelles.

The solubility limit of cholesterol in micellar taurocholate-lysolecithin cholesterol mixtures depends on lysolecithin concentration. It is 0.21 mM of cholesterol for 2.1 mM of lysolecithin, 0.27 mM for 3.30 mM, 0.35 mM for 4.40 mM and 0.60 mM for 9.00 mM. The solubility remains lower than in the case of lecithins in comparable molar ratios.

Concerning the micellar size, Figure 1 shows the variation of micellar size related to lecithin or lysolecithin concentrations. The slopes of these two curves are very different and show that in the case of lysolecithins the size of the micelles does not change significantly when the lysolecithin concentration increases. In all cases, the size of mixed micelles containing lysolecithins is smaller than that of lecithin micelles in comparable molar ratios.

It is likely that the structure of this type of mixture is similar to that obtained with lecithins. Lysolecithins are more water soluble than lecithins and are distributed between the micellar and aqueous phase in the presence of bile salts. Solubilized cholesterol, however, must be completely inside the bile salt-lysolecithin-cholesterol micelles. It appears that lysolecithins alone can incorporate cholesterol only in an insoluble lamellar phase at a lysolecithin/cholesterol ratio 1/1 (19). In the presence of bile salts, lysolecithins and cholesterol are found in the micellar phase (20).

The results of cholesterol uptake from bile salt-lysolecithin mixed micelles are given in Table 3. It appears that cholesterol uptake depends on both cholesterol saturation of the micelle and on micellar size.

From micelles of small size (lysolecithin between 2.1 and 4.4 mM), cholesterol uptake seems to depend on cholesterol saturation alone. For a given cholesterol concentration (0.21 mM) when lysolecithin concentration increases from 2.1 to 3.3 mM, micellar size is unchanged, and uptake and saturation of cholesterol vary in parallel: cholesterol uptake decreases by 19% and cholesterol saturation by 22%. Similarly, cholesterol saturation decreases by 40% and cholesterol uptake by 43% when lysolecithin concentration increases from 2.1 to 4.4 mM.

For higher lysolecithin concentration (9.0 mM), cholesterol uptake decreases in spite of a

TABLE 3

Effect of Varying Concentrations of Lysolecithin on Cholesterol Uptake

Concentration in micellar mixtures					
Lysolecithin (mM)	Cholesterol (mM)	Cholesterol saturation (%)	n	Cholesterol uptake (nmoles·100mg^{-1}·5min^{-1})	Apparent adherent fluid volume (μl·100mg^{-1}·5min^{-1})
2.1	0.21	100	8	21.96 ± 0.96	57.45 ± 3.81
3.3	0.21	78	8	16.64 ± 1.64	61.81 ± 3.11
4.4	0.21	60	7	12.47 ± 1.67	56.81 ± 3.68
9.0	0.60	100	11	12.21 ± 1.17	55.43 ± 5.39

The taurocholate concentration is kept constant at 10 mM.
Values are mean ± SE. n = number of animals.

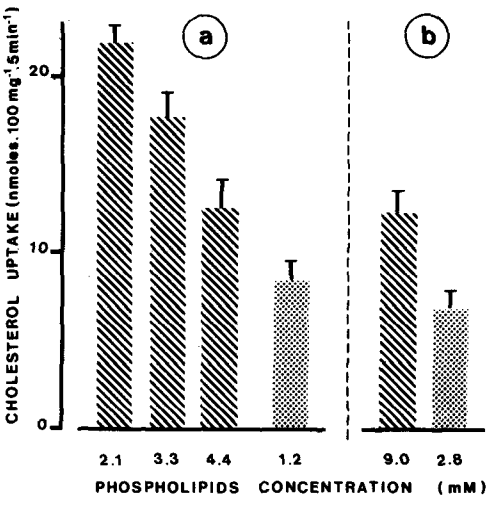

FIG. 2. Effect of lysolecithin (▒▒) and lecithin (▨) on cholesterol uptake. For all micelles, the bile salt concentration is kept constant (10 mM). In part (a), the cholesterol concentration is 0.21 mM for all mixtures and the apparent molecular weight is about 50,000. In part (b), for the two micelles, the cholesterol concentration is 0.54 mM and the size is 70,000. It must be emphasized that cholesterol content of the micelles in parts a and b is very different. This explains why cholesterol uptake from phospholipid micelles is not very much decreased in b compared to a, in spite of the larger size of the micelles.

higher cholesterol content (0.6 mM). This is consistent with the corresponding results with lecithins, showing the importance of micellar size.

Comparative Effect of Lecithins and Lysolecithins on Intestinal Cholesterol Uptake

A comparison of Tables 1 and 3 shows that cholesterol uptake is generally higher in the presence of lysolecithins than lecithins. From this general result, it is difficult to be certain if the difference is due only to a change of micellar size and cholesterol saturation degree or to a specific influence of lecithins on cholesterol uptake. To answer this question, it is necessary to compare cholesterol uptake in two groups of micelles, similar in size and in quantity of cholesterol. This is obtained in two cases.

1) Figure 2a shows the result obtained from mixed micelles containing either lecithins (1.2 mM) or lysolecithins (2.1 mM to 4.4 mM). Both series are small size micelles (50,000) and contain the same amount of cholesterol (0.21 mM). In this case, cholesterol uptake is significantly higher (+48%) in the presence of lysolecithins (4.4 mM) than in the presence of lecithins ($2p \leq 0.01$). This difference is even greater (+160%) if we compare this phenomenon with lysolecithin mixed micelles with the same saturation degree as lecithin (2.1 mM lysolecithin versus 1.2 mM lecithin). The comparison between the effects of lecithin (1.2 mM) and lysolecithin (3.3 mM) on cholesterol uptake after an incubation time from 3 to 15 min is performed to check the linearity of the results (see methods). At 15 min cholesterol uptake is 1.90-fold greater in the presence of lysolecithin, this ratio remaining constant after 3 and 5 min.

2) Figure 2b shows the result from mixed micelles with a larger micellar size (70,000) which is obtained with 2.1 mM lecithin and 9 mM lysolecithin. Both exhibit the same size, the same cholesterol saturation and the same quantity of cholesterol (0.54 mM). Cholesterol uptake from lysolecithin micelles is significantly higher (77%) than in the presence of lecithin. This shows that the effect of lecithins on the size of mixed micelles and their degree of cholesterol saturation is not sufficient to explain their effect on intestinal absorption of lipids.

The greater uptake from lysolecithin micelles cannot result from a breakdown of the intestinal barrier due to a detergent effect of lysolecithin (21) for several reasons. The lysolecithin concentration we used in our experiments was much lower than that which Bolin et al. (22) considered as having a damaging effect on the intestinal membrane. To confirm this observation in presence of bile salt, we have measured the diffusion volume of ^{3}H-inulin. In the case of intact membrane, ^{3}H-inulin does not cross the enterocyte membrane, and the diffusion volume corresponds to adherent fluid in contact with the membrane. In the case of modifications of membrane permeability, the diffusion volume will include the adherent fluid plus ^{3}H-inulin uptake and will appear increased. The results of adherent fluid volumes are shown in Tables 1 and 3. Whatever the phospholipid concentrations, there is no significant difference between lecithin and lysolecithin. Furthermore, the average of adherent fluid volumes are the same as those determined from an incubation medium containing ^{3}H-inulin without micelles ($58.00 \pm 3.84\ \mu l \cdot 5min^{-1}$). In all of our experimental conditions, the results cannot be influenced significantly by changes of membrane permeability.

Lecithin and Lysolecithin Uptake from Mixed Micelles

The differential behavior of lysolecithins and lecithins nevertheless remains explicable without special mechanisms for cholesterol uptake occurring, if we consider the relative rates of absorption of lecithins and lysolecithins. We measured the uptakes of lysolecithins and lecithins in the same experimental conditions as used for cholesterol. We used ^{14}C-lecithin or

^{14}C-lysolecithin and ^{3}H-inulin as radiolabeled volume marker. The comparison is made with mixed micelles containing 0.21 mM of cholesterol and 1.2 mM lecithin or 4.4 mM lysolecithin. Lecithin uptake (20.11 ± 3.77 nmoles · 100 mg^{-1} · 5 min^{-1} for 24 experiments) is very slight in comparison to that of lysolecithin (345 ± 14 nmoles · 100 mg^{-1} · 5 min^{-1} for 12 experiments). This difference is highly significant ($2p \leqslant 0.01$). As shown above, lysolecithin absorption is not due to a possible membrane injury. We studied the influence of the presence of lysolecithins on lecithin uptake. Lecithin absorption is unchanged when studied in the presence of 9.0 mM of lysolecithin (18.20 ± 1.80 nmol·5 min^{-1} instead of 21.93 ± 2.83 nmol·5 min^{-1} without lysolecithin).

The observed difference between lysolecithin and lecithin uptake seems to explain the results, if we consider that cholesterol absorption is a simple partitioning between a saturated micellar phase and an aqueous phase in contact with the epithelium. In presence of lecithin, these micelles should remain stable with no tendency toward supersaturation with cholesterol, because the lecithins are not absorbed to a great extent. As a result of the considerable absorption of lysolecithins, on the other hand, the bile salt-lysolecithin-cholesterol micelles should tend toward supersaturation with cholesterol leading to cholesterol absorption. The dissociation of mixed micelles in different components during absorption after diffusion through the unstirred water layer seems to be a determinant factor for rate of cholesterol uptake (23). The release of lysolecithin and its uptake lead to a cholesterol supersaturation, located near the membrane; this behavior does not occur with lecithins which are not absorbed.

Our results are thus compatible with the hypothesis that lecithins decrease lipid absorption by modifying the equilibrium and the stability of mixed micelles.

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Fatty Acid Metabolism and Cell Proliferation. VII. Antioxidant Effects of Tocopherols and Their Quinones

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ABSTRACT

The antioxidant capacities of α- and γ-tocopherols (α-E and γ-E) and their quinones (α-EQ and γ-EQ) were determined in non-biological and biological systems. The non-biological system consisted of arachidonic acid [20:4 (n-6)], the oxidant cumene hydroperoxide, and a Fe^{3+} catalyst to facilitate malondialdehyde (MDA) formation from lipid peroxides. α-E and γ-E had similar antioxidant capacities in this sytem. α-EQ also functioned as an antioxidant, while γ-EQ exhibited a crossover effect by functioning as an antioxidant at low concentrations and a prooxidant at high concentrations. Biological lipid peroxidation in smooth muscle cells challenged with 20:4 (n-6) was measured both by MDA formation in confluent cultures and by cell growth in proliferating cultures. α-E, γ-E and α-EQ had similar antioxidant capacities, but γ-EQ was highly cytotoxic for cells in both confluent and proliferating cultures. Cellular retention of antioxidants was estimated indirectly from MDA formation when cells were loaded with an antioxidant (preincubation) and then incubated for varying periods of time in fresh media containing 20:4 (n-6). Cellular retention also was measured directly with tritiated α-E and tritiated α-EQ. These studies showed that cellular retention decreased in the sequence γ-E>α-E> α-EQ. Thus, cellular retention does not explain the enhanced antioxidant capacity of α-E compared to γ-E that has been reported for animal systems. The antioxidant capacity of α-E evidently is enhanced by its metabolism to a quinone which, unlike the quinone from γ-E, functions as a biological antioxidant.

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INTRODUCTION

Previous studies in this series have focused on lipid peroxidation in cultured cells and its effect on cell proliferation. In the present investigation, tissue culture is used to compare the biological properties of naturally occurring tocopherols and their quinone metabolites.

The relative antioxidant capacities of α-tocopherol (α-E) and γ-tocopherol (γ-E) differ in biological and non-biological systems. Early studies generally concluded that γ-E was more effective than α-E in preventing non-biological lipid peroxidation (1,2). However, relative antioxidant effectiveness depended on the specific oxidizing system. For example, γ-E is much more effective than α-E in preventing the peroxidation of lard at 100 C (1), yet Burton and Ingold (3) measured the rate constant for abstraction by peroxyl radical of the phenolic hydrogen and found that in this sytem α-E actually was slightly more reactive than γ-E. In contrast to non-biological systems, a number of early studies showed that α-E was far better than γ-E as a biological antioxidant (1,2). The α-E had much greater activity than γ-E in biological systems associated with a vitamin E deficiency such as fetal resorption, red cell hemolysis and muscular dystrophy (4). A recent study found that γ-E was only 37% as effective as α-E in reducing lipid peroxidation (pentane production) by iron-loaded rats (5).

Several investigators suggested that the difference in biopotency of α-E and γ-E was explained by differences in the tissue retention of the two compounds (2,6,7). Studies investigating α-E and γ-E found no significant differences in absorption, plasma transport or tissue uptake of these agents (8,9). Since the difference in biopotency of α-E and γ-E was found within minutes after intraperitoneal injection (10), Peake et al. (9) suggested that either intracellular binding or metabolism could be important in determining tocopherol activity.

The difference in biopotency of α-E and γ-E may be explained by differences in their conversion to quinones and the biological properties of these metabolites. Many studies show that α-E is metabolized to α-tocopherylquinone (α-EQ) (6-8,11-13). We found that α-E and α-EQ were both effective antioxidants in tissue culture and that high concentrations of α-EQ were less cytotoxic than α-E when they were added to cells in culture (14-19). Additionally, several early investigators reported that α-EQ, although less effective than α-E, prevented and cured the creatinuria, paralysis, weight loss and fetal resorbtion that are characteristic of the vitamin E deficiency state (20,21). The duration of the antioxidant effect is much shorter with α-EQ

than with α-E, suggesting a difference may occur in the cellular retention of these two agents (20). The metabolism of γ-E differs significantly from α-E. The majority of γ-E is converted to metabolites such as the dimer, and little γ-E, unlike α-E, is converted to γ-tocopherylquinone (γ-EQ) (8). Furthermore, γ-EQ has a different one electron redox potential than α-EQ and little is known about the antioxidant capacity and other biological properties of γ-EQ that could be affected by its redox potential.

The studies summarized above suggest that differences, in vivo, between the antioxidant capacities of α-E and γ-E may be related either to their retention in cells or to their conversion to quinones. These studies also suggest that differences, in vivo, between the antioxidant capacities of α-E and α-EQ may be related to their retention in cells. In this study, we examine the cellular retention of α-E, γ-E and α-EQ, and because little previous work has been reported, we investigate the role of γ-EQ as a biological antioxidant.

MATERIALS AND METHODS

Materials

Arachidonic acid [20:4 (n-6)] was purchased from NuChek Prep (Elysian, Minnesota), purified by elution from a Unisil® column with hexane/ether (9:1, v/v), and used only when thin layer chromatography showed that lipid peroxides were absent (22). α-E, γ-E and α-EQ were purchased from Eastman Organic Chemicals (Rochester, New York). D-α-[^{3}H]-Tocopherol (24 ci/mmole) was purchased from Amersham International (Arlington Heights, Illinois). The tritiated α-E was analyzed by HPLC and found to be partially oxidized to a mixture of α-E and α-EQ. Labeled α-EQ (elution time 3.9 min) and labeled α-E (elution time 5.1 min) were separated by elution from an Altex Ultrasphere ODS (4.6 × 250 mm) column with 100% methanol at a flow-rate of 1.5 ml/min. α-EQ was purified by elution from an Ultrasphere ODS (4.6 × 550 mm) column.

γ-EQ was synthesized from γ-E by $FeCl_3$ oxidation (23), and purity was established by HPLC in two systems: System I, elution from a Nucleosil 5 C-18 (4.6 × 150 mm) column with methanol:water (95:5) at a flow-rate of 1.5 ml/min; System II, elution from a Nucleosil 50-5 (4.6 × 250 mm) column with isopropyl ether:hexane (9:1) at a flow-rate of 2.0 ml/min. Purity was 98.3% in System I (elution time 5.21 min) and 98.8% in System II (elution time 6.78 min). No single impurity exceeded 0.5%.

Non-Biological Lipid Peroxidation

The model system which has been described previously (18) contained 1 mM 20:4 (n-6) in 0.1 M phosphate buffer (pH 7.4) 2.84 mM cumene hydroperoxide (CHP) and 41 μM Fe^{3+} in a final volume of 1.2 ml. Mixtures were incubated at room temperature for 10 min. Lipid peroxides were assayed by a TBA procedure (14,17,18). In this procedure, the fatty acid which imparted turbidity to the acidified (TBA) solution was extracted with chloroform:acetic acid (2:1, v/v) before the absorbance at 532 nm was measured. Absorbance data are reported as nmoles of malondialdehyde (MDA).

Tissue Culture

Primary cultures of smooth muscle cells were established from the dissected medial layer of guinea pig aorta from prepubertal males (24). The medium for growing cells to confluency (Growth Medium), the medium in lipid peroxidation studies with confluent cells (Experimental Medium) and Cloning Medium have been described (18). Media were supplemented with fetal bovine serum (Sterile Systems, Logan, Utah; Hyclone lots 100348 and 010439). Cells from random confluent cultures were detached with trypsin and counted (25). Cells were used at passage levels 4 to 6. 20:4 (n-6), tocopherols and tocopherylquinones were dissolved in 95% ethanol and diluted with Experimental Medium or Cloning Medium. Control culture were treated with medium containing the same amount of 95% ethanol.

Biological Lipid Peroxidation

The intracellular lipid peroxidation of cells in tissue culture (14,17,18) was measured with cells seeded at 1.3×10^4 cells/cm^2 in flasks containing 4 ml of Experimental Medium. The cells were grown to confluency before treatments were initiated. Lipid peroxides, which were found only in bound intracellular lipids (14,17,18), were measured at the end of the treatment period by a TBA procedure as previously described (14,17,18). Lipid peroxides are reported as nmoles of MDA/culture.

Cellular Retention of Tritiated α-E and α-EQ

Confluent cultures, preincubated for 24 hr with 20 μM α-E or 10 μM α-EQ, incorporated similar amounts of these compounds in cells (4.3 and 3.5 nmoles/culture, respectively). Cellular uptake was estimated from the α-E or α-EQ remaining in incubation media and media from one rinse. The labeled cells were incubated in fresh media for an additional 24 hr and total radioactivity released into the media was mea-

TABLE 1

Effect of Tocopherols and Their Quinones on Lipid Peroxidation (TBA Test) in the Non-Biological Model System

System	MDA[c]
20:4 (n-6) alone[a]	0.92 ± 0.08 (17)
Model system[b]	2.76 ± 0.06 (34)
+ 1 μM α-E	1.39 ± 0.06 (11)[d]
+ 10 μM α-E	0.72 ± 0.02 (11)[d]
+ 100 μM α-E	0.72 ± 0.03 (11)[d]
+ 1000 μM α-E	0.89 ± 0.04 (10)[d]
F ratio	325
+ 1 μM γ-E	1.55 ± 0.05 (6)[d]
+ 10 μM γ-E	0.91 ± 0.02 (6)[d]
+ 100 μM γ-E	0.67 ± 0.03 (6)[d]
+ 1000 μM γ-E	0.59 ± 0.01 (6)[d]
F ratio	240
+ 1 μM α-EQ	1.83 ± 0.06 (8)[d]
+ 10 μM α-EQ	0.93 ± 0.03 (8)[d]
+ 100 μM α-EQ	0.63 ± 0.02 (8)[d]
+ 1000 μM α-EQ	0.63 ± 0.02 (8)[d]
F ratio	295
+ 1 μM γ-EQ	1.88 ± 0.07 (7)[d]
+ 10 μM γ-EQ	1.41 ± 0.02 (10)[d]
+ 100 μM γ-EQ	1.93 ± 0.06 (10)[d]
+ 1000 μM γ-EQ	3.09 ± 0.08 (8)
F ratio	94.8

[a]1 mM 20:4 (n-6) in phosphate buffer.

[b]The model system contained 1 mM 20:4 (n-6), 2.84 mM CHP and 41 μM Fe^{3+} in phosphate buffer.

[c]MDA [nmol/μmol 20:4 (n-6)]; mean ± SEM; number of experiments in parenthesis.

[d]Differed significantly from the Model system (Scheffe test).

sured using Aqueous Counting Scintillant II (Amersham). Two volumes of ethanol were added to media or cells and the mixture extracted twice with 4 volumes of hexane. Hexane extracts were combined, α-E and α-EQ separated by HPLC (Ultrasphere ODS column) and counted.

Cell Proliferation

Cell proliferation was measured in Falcon plates as previously described (15-18,26,27). Smooth muscle cells, 3-5 days postconfluent, were seeded at low densities. A relative cell count was obtained from the total cell area on the Falcon plate. Total cell area was measured by image analysis (15-18,26,27) using an Optomax Visual Analysis System (Optomax, Wallis, New Hampshire). Cells from the same primary culture and the same batch of growth medium were compared in each treatment group.

Statistics

Data are reported as mean ± SEM. The significance of differences in a treatment series was determined by a one-way analysis of variance (F-ratio). Individual treatments were compared with the control by the Tukey-HSD test (sample sizes equal) or the Scheffe test (sample sizes unequal).

RESULTS

Non-Biological Lipid Peroxidation

Fatty acid peroxides (TBA reaction) were detected when 20:4 (n-6) was incubated for 10 min in a phosphate buffer at pH 7.4 (Table 1). The peroxide yield tripled when the oxidizing agent CHP and a Fe^{3+} catalyst were added to the incubation system. Both α-E and γ-E partially inhibited the oxidation reaction at concentrations as low as 1 μM and these agents blocked the CHP-Fe^{3+} oxidation reaction at 10 μM and higher concentrations. Little difference was detected between the antioxidant capacities of α-E and γ-E in the model system.

Tocopherylquinones acted in a different manner (Table 1). Little difference was detected between α-EQ and either α-E or γ-E in the model system. On the other hand, γ-EQ inhibited the oxidation reaction at low concentrations, but the inhibitory effect with γ-EQ diminished as its concentration increased, leading to enhanced overall oxidation with 1000 μM γ-EQ.

Biological Lipid Peroxidation

Biological lipid peroxidation was measured both by intracellular MDA formation (14,17,18) in confluent cultures and by cell growth in proliferating cultures. In the first study, α-E and γ-E showed similar antioxidant capacities for blocking MDA formation in cells challenged with 120 μM 20:4 (n-6) (Table 2). Tocopherols in these experiments functioned as antioxidants; other studies have shown that agents such as α-E have no effect on the uptake and distribution of 120 μM 20:4 (n-6) in confluent smooth muscle cells (28,29).

Tocopherylquinones again acted in a different manner (Table 2). MDA formation was inhibited as effectively by α-EQ as by tocopherols. However, large numbers of cells detached from the culture flask, indicating cell death when γ-EQ was added to the cultures.

Previous studies from our laboratory (14-19, 27,29,30) have shown that antioxidants such as α-E, α-EQ, α-napthol, nordihydroguaiaretic acid, propyl gallate and dipyridamole promoted growth in cell cultures challenged with 20:4 (n-6). When tocopherols were compared in this system, cell proliferation was stimulated even more effectively with the antioxidant γ-E than

TABLE 2

Effect of Tocopherols and Their Quinones on Lipid Peroxidation (TBA Test) in Confluent Cultures of Aorta Smooth Muscle Cells Incubated with 20:4 (n-6) for 24 hr

Treatment[a]	MDA[b]
120 μM 20:4 (n-6) alone	16.1 ± 0.40 (10)
+ 10 μM α-E	0.48 ± 0.22 (4)[c]
+ 25 μM α-E	0.48 ± 0.10 (4)[c]
F ratio	544
+ 10 μM γ-E	0.85 ± 0.10 (4)[c]
+ 25 μM γ-E	0.63 ± 0.07 (4)[c]
F ratio	545
+ 10 μM α-EQ	0.52 ± 0.09 (4)[c]
+ 25 μM α-EQ	0.29 ± 0.14 (4)[c]
F ratio	565
+ 10 μM γ-EQ	cell death[d]
+ 25 μM γ-EQ	cell death[d]

[a]20:4 (n-6) and antioxidants were added together in fresh media to confluent cultures.

[b]MDA in nmol/cultures; mean ± SEM; number of experiments in parenthesis.

[c]Differed significantly from 20:4 (n-6) alone (Scheffe test).

[d]Cells detached from culture flask.

TABLE 3

Effect of Tocopherols and Their Quinones in Cell Proliferation with Aorta Smooth Muscle Cells

Treatment[a]	Relative cell number (%)
60 μM 20:4 (n-6)	100 ± 5.5 (14)
+ 1 μM α-E	128 ± 6.6 (14)[c]
+ 10 μM α-E	103 ± 5.6 (13)
F ratio	6.8
+ 1 μM γ-E	156 ± 6.3 (14)[c]
10 μM γ-E	123 ± 8.9 (13)
F ratio	16.8
+ 1 μM α-EQ	146 ± 5.6 (13)[c]
10 μM α-EQ	119 ± 3.5 (13)[c]
F ratio	22.1
+ 1 μM γ-EQ	cell death[d]
+ 10 μM γ-EQ	cell death[d]

[a]20:4 (n-6) and antioxidants were added together in fresh media to proliferating cultures.

[b]Mean ± SEM; number of experiments in parenthesis.

[c]Differed significantly from 20:4 (n-6) alone (Tukey-HSD test).

[d]Variable cell growth with 1 μM γ-EQ; less than 10% cell growth with 10 μM γ-EQ.

with α-E (Table 3). The cytotoxicity of γ-EQ in confluent cultures (Table 2) was again apparent in cell proliferation studies. Thus α-EQ enhanced cell growth while the same concentrations of γ-EQ caused extensive cell death (Table 3).

Cellular Retention of Tocopherols and α-Tocopherylquinone

Cellular retention of α-E, γ-E and α-EQ was estimated using confluent cultures. Cells initially were loaded with an antioxidant by preincubation in media containing the antioxidant. Cells then were incubated in fresh media that contained 20:4 (n-6) but no antioxidant. In these experiments, cells that retained more antioxidant would be expected to generate proportionally less intracellular MDA when they were challenged with 20:4 (n-6) in the fresh media.

Cellular retention varied with the specific antioxidant. Less MDA was formed in cells preincubated with 10 μM γ-E than 10 μM α-E, indicating that more γ-E than α-E was retained in the cells (Table 4). At a 10 μM concentration, α-EQ was retained even less effectively than α-E. Thus, MDA in cells preincubated with 10 μM α-EQ did not differ significantly from MDA in cells preincubated with media alone (Table 4).

Cellular retention was a function of the antioxidant concentration during the preincubation stage of the experiment. When cells were preincubated with 50 μM antioxidant, sufficient α-E was retained to block completely MDA formation, and sufficient α-EQ was retained to block significantly MDA formation (Table 4).

Cellular retention was a function of the incubation time for cells in fresh media. When cells

TABLE 4

Cellular Retention of Tocopherols and α-Tocopherylquinone Measured by Lipid Peroxidation (TBA Test) in Confluent Cultures of Aorta Smooth Muscle Cells Incubated with 20:4 (n-6) for 24 hr

Treatment[a]	MDA[b]
A. 120 μM 20:4 (n-6)	18.5 ± 1.6 (6)
+ 10 μM α-E	10.3 ± 2.0 (7)[c]
+ 10 μM γ-E	4.6 ± 0.7 (6)[c]
+ 10 μM α-EQ	15.5 ± 1.5 (7)
B. 120 μM 20:4 (n-6)	18.7 ± 0.7 (12)
+ 50 μM α-E	0.7 ± 0.3 (12)[c]
+ 50 μM α-EQ	2.3 ± 0.9 (12)[c]

[a]Confluent cultures were preincubated for 48 hr with antioxidants; 20:4 (n-6) was then added with a complete media change, and cells were incubated for an additional 24 hr. Experiments A and B used different lots of fetal bovine serum.

[b]MDA in nmol/culture; mean ± SEM; number of experiments in parenthesis.

[c]Different significantly from 20:4 (n-6) alone (Tukey-HSD test).

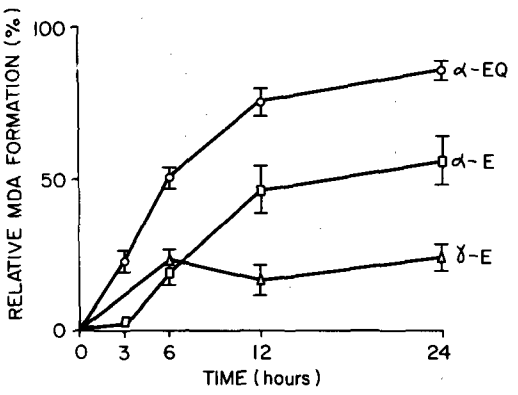

FIG. 1. Retention of Antioxidants by cells preincubated with 10 μM antioxidant to incorporate antioxidant in cells and then incubated with 120 μM 20:4 (n-6) in fresh media without antioxidant for different periods of time. Data (mean ± SEM in %) represent MDA in cells pretreated with antioxidant relative to cells pretreated with media alone. The relative MDA values at 24 hr are calculated from the actual MDA values reported in Table 4.

TABLE 5

Cellular Retention after a 24 hr Incubation Period of Tritiated α-Tocopherol and α-Tocopherolquinone in Confluent Cultures of Aorta Smooth Muscle Cells

	Preincubation (cpm in %)	
Distribution	α-E	α-EQ
Released to media		
α-E	16[a]	0
α-EQ	16	65
Retained in cells		
α-E	52	0
α-EQ	16	35

[a]Average of 2 experiments.

were preincubated with 10 μM antioxidant, the difference in MDA yield between α-E and γ-E was apparent only after cells were incubated in fresh media for 12 hr (Fig. 1). On the other hand, 10 μM α-EQ was less effective than the tocopherols in blocking MDA when cells were incubated for as little as 3 hr with fresh media (Fig. 1). These data (Table 4 and Fig. 1) show that the cellular retention of the antioxidants decreases in the order γ-E>α-E>α-EQ.

Since MDA is only an indirect measure of cellular retention, tritiated α-E and α-EQ were used as a direct measure of cellular retention. Similar amounts of α-E and α-EQ were incorporated in confluent cells (see Methods). The amount of α-EQ released from cells labeled with α-EQ was much greater than the combined amount of α-E and α-EQ released from cells labeled with α-E (Table 5). Furthermore, α-E was converted to α-EQ in cells labeled with α-E, and the α-E/α-EQ ratio was higher in cells than in media. These data show that α-EQ is released more rapidly than α-E from cells, and these data are consistent with MDA data indicating that cellular retention decreases in the order α-E>α-EQ.

DISCUSSION

Large differences in antioxidant capacity have been reported for naturally occurring tocopherols in biological and non-biological systems (1-10). Our data show that α-E and γ-E have similar antioxidant capacities in a non-biological model system utilizing a polyunsaturated fatty acid, CHP, as the oxidizing agent, and a Fe^{3+} catalyst for the decomposition of fatty acid peroxides to MDA (Table 1). These results support the observation of Burton and Ingold (3) that the relative antioxidant capacities of tocopherols depend on the nature of the oxidizing agent in the non-biological oxidizing system.

The antioxidant capacities of α-E and γ-E also were measured in a biological model system utilizing a polyunsaturated fatty acid and smooth muscle cells in tissue culture. Two criteria, MDA formation and cell proliferation, were used to evaluate antioxidant capacity in the biological system. The results showed that α-E and γ-E have similar antioxidant capacities in cell cultures (Table 2 and 3).

The antioxidant capacities of α-E and γ-E in animals may reflect differences either in cellular retention or in metabolism (9). Tissue cultures may be used to measure cellular retention when the cells are first loaded with antioxidant (preincubation) and then challenged by incubation with a polyunsaturated fatty acid in fresh media. Since these experiments (Table 4 and Fig. 1) showed that γ-E was retained in smooth muscle cells more effectively than α-E, it is unlikely that differences in the antioxidant capacities of α-E and γ-E in animals are related to cellular retention.

Tocopherols are metabolized to quinones, dimers and a number of acid derivatives resulting from ω-oxidation and subsequent β-oxidation of the isoprenoid side chain (7,31). Studies from our laboratory (14-19) and elsewhere (20, 21) have shown that α-EQ, the quinone metabolite of α-E, functions as an antioxidant. We suggested that α-EQ was reduced to its semiquinone by superoxide and that the semiquinone actually functioned as the antioxidant in chain termination (14,19). This concept is supported by studies showing the reduction of quinones

to semiquinones with superoxide (32,33) and by the recent observation of Ozawa and Hanaki (34) that α-EQ is reduced to its semiquinone by superoxide.

The reduction of a quinone to the semiquinone depends on its one electron redox potential. The substituted para quinones, α-EQ and γ-EQ, have different redox potentials, and these antioxidants function differently in the non-biological model system (Table 1). One quinone, α-EQ, acted as an antioxidant throughout a wide concentration range while the other quinone, γ-EQ, acted first as an antioxidant and then as a prooxidant at higher concentrations. Thus, γ-EQ data show the "crossover" effect that has been reported for a number of antioxidants (35-37).

The difference between γ-EQ and α-EQ was most apparent in the biological model system where the addition of γ-EQ to tissue cultures led to extensive cell death (Table 2 and 3). Peake and Bieri (8) found that γ-E was converted preferentially to the dimer in animals, while α-E was preferentially converted to the quinone. This difference in metabolism may explain both why γ-E is not toxic and why γ-E is less effective than α-E as a biological antioxidant. The metabolism of γ-E to a dimer rather than quinone prevents toxicity, while the metabolism of α-E to a quinone enhances antioxidant capacity. This explanation is supported by the observation that δ-E, the most effective antioxidant in non-biological systems and the least effective tocopherol antioxidant in animal systems (1,2), is converted in animals to an ω and β oxidation product, not to the quinone (31). δ-E and γ-e are less readily oxidized than α-E to quinones, even in non-biological systems (37).

Mackenzie et al. (20) first suggested that differences, in vivo, between the antioxidant capacities of α-E and α-EQ could be related to cellular retention. This hypothesis is supported by the present study which showed that α-EQ had the lowest cellular retention when it was compared to α-E or γ-E (Table 4 and 5, and Fig. 1). Thus, α-EQ will function as an antioxidant only when it is generated in situ or administered over short time intervals. These data suggest that animals maintained on an α-E free diet supplemented with injected α-EQ might develop rapidly a vitamin E deficiency when α-EQ is withdrawn, and as a consequence α-EQ might be used to produce a vitamin E deficiency in relatively healthy animals. Poor cellular retention explains why α-EQ does not accumulate in tissues even though α-E is rapidly metabolized to α-EQ in most tissues after the oral administration of its esters (38).

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Inhibition of C_{28} and C_{29} Phytosterol Metabolism by N, N-Dimethyldodecanamine in the Nematode *Caenorhabditis elegans*

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ABSTRACT

Effects on the metabolism of campesterol and stigmasterol in *Caenorhabditis elegans* were investigated using N,N-dimethyldodecanamine, a known inhibitor of growth, reproduction and the Δ^{24}-sterol reductase of this nematode. 7-Dehydrocholesterol was the predominant sterol (51%) of *C. elegans* grown in stigmasterol-supplemented media, whereas addition of 25 ppm amine resulted in a large decrease in the relative percentage of 7-dehydrocholesterol (23%) and the accumulation of a substantial proportion (33%) of Δ^{24}-sterols (e.g., cholesta-5,7,24-trienol) and $\Delta^{22,24}$-sterols (e.g., cholesta-5,7,22, 24-tetraenol) but yielded no Δ^{22}-sterols. Dealkylation of stigmasterol by *C. elegans* proceeded in the presence of the Δ^{22}-bond; reduction of the Δ^{22}-bond occurred prior to Δ^{24}-reduction. Addition of 25 ppm amine to campesterol-supplemented media altered the sterol composition of *C. elegans* by increasing the percentage of unmetabolized dietary campesterol from 39 to 60%, decreasing the percentage of 7-dehydrocholesterol from 26 to 12%, and causing the accumulation of several Δ^{24}-sterols (6%). *C. elegans* also was shown to be capable of dealkylating a $\Delta^{24(28)}$-sterol as it converted 24-methylenecholesterol to mostly 7-dehydrocholesterol. The proposed role of 24-methylenecholesterol as an intermediate between campesterol and 7-dehydrocholesterol was supported by the results.
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INTRODUCTION

One of our research goals is the discovery of models for novel chemical control agents against parasitic nematodes of agricultural importance. This requires the acquisition of fundamental knowledge concerning the physiological and biochemical effects of such chemicals on nematodes. We have found that a number of compounds exhibiting nematicidal activity also disrupt sterol metabolism in nematodes, insects and other animals. Steroid metabolism thus may be a viable target for selective nematicidal agents.

Nematodes possess a nutritional requirement for sterol (1-4); they are unable to biosynthesize sterols de novo (5-8), yet are capable of metabolizing exogenous sterols. The free-living nematode *Caenorhabditis elegans* has the ability to remove (dealkylate) the 24-alkyl substituent, whether it be an α-ethyl, α-methyl, or β-methyl group, from a variety of dietary 24-alkylsterols (9-12). In only one of these cases, i.e., using dietary sitosterol, has the metabolic pathway of dealkylation been elucidated (10,11); several dealkylation inhibitors were used to accumulate sterols postulated as intermediates in the metabolism of sitosterol. Based on these accumulated intermediates and the site of enzymatic inhibition, the mechanism of sitosterol dealkylation in *C. elegans* appears to be very similar to that in certain phytophagous insects (13,14).

Whereas examination of the dealkylation mechanism in insects has been extended to include other 24-alkylsterols such as campesterol or stigmasterol (13-17), a comparative investigation of their dealkylation in *C. elegans* has not been accomplished. Therefore, in the present study, *C. elegans* was propagated in media supplemented with a 24-alkylsterol, either campesterol (24α-methylcholesterol) or stigmasterol (24α-ethylcholesta-5,22E-dienol), alone or in combination with N,N-dimethyldodecanamide or N,N-dimethyldodecanamine, compounds which inhibit population growth of *C. elegans* (11). The inhibitory amine previously was shown to disrupt the sitosterol dealkylation process in *C. elegans* (11) and in certain insects (18,19) via Δ^{24}-sterol reductase inhibition. Dietary 24-methylenecholesterol also was used in this study, since it was postulated as an intermediate in the conversion of campesterol to cholesterol by *C. elegans* (12). The identifications of sterol compositions of *C. elegans* propagated in these media have permitted us to postulate dealkylation pathways and to compare these pathways in *C. elegans* with those that occur in insects.

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MATERIALS AND METHODS

C. elegans was cultured axenically in a semi-defined aqueous medium as previously described (11) and supplemented with sterol at a concentration of 25 ppm. Dietary campesterol, which contained about 3% sitosterol as an impurity by gas-liquid chromatography (GLC), was isolated from soybean sterols by fractional crystallizations from acetone. Nonradiolabeled stigmasterol (>99% purity by GLC) was a gift from Upjohn Company (Kalamazoo, Michigan). 24-Methylenecholesterol (>99% purity by GLC) was obtained from bee-collected almond pollen and purified by argentation column chromatography. [2,4-^{3}H]Stigmasterol was prepared according to Thompson et al. (20) and, after column-chromatographic purification, possessed a radiochemical purity greater than 99% by GLC and thin-layer chromatography (TLC), and was utilized at a specific activity of 3600 dpm/μg (0.67 Ci/mol). N,N-Dimethyldodecanamine was commercially available from Ethyl Corporation (Baton Rouge, Louisiana) as ADMA 2. N,N-Dimethyldodecanamide was prepared as described previously (18). Each compound was solubilized simultaneously with the dietary sterol.

Extraction and separation of total sterols (after saponification) from lyophilized nematodes (harvested after two weeks) and acetylation of isolated sterols were performed as before (11). Quantitative and qualitative analyses were done by GLC; relative retention times (RRTs) were measured relative to cholesterol (for free sterols) or cholesteryl acetate (for steryl acetates). Final identifications were confirmed by gas chromatography-mass spectrometry (GC-MS) and ultraviolet (UV) spectroscopy. GLC was performed using the following chromatographic systems: coiled glass columns packed with 2.0% OV-17 or 2.0% SE-30, and a J & W DB-1 fused silica capillary column. Distribution of radioactivity was determined by radioassay of fractions from column chromatography, TLC, and GLC effluent. Additional instrumental details have been described earlier (11).

RESULTS

Metabolism of Stigmasterol

C. elegans, when propagated in media containing stigmasterol alone, contained, excluding dietary sterol, predominantly 7-dehydrocholesterol with lesser amounts of cholesterol, lathosterol, cholesta-5,7,9(11)-trienol, 4α-methylcholest-8(14)-enol, and 4α-methylcholest-7-enol (lophenol) (Table 1). Analytical properties (GLC, GC-MS, UV absorbance) of these sterols were identical to those of authentic standards.

TABLE 1

Relative Percentages of Total Sterols from *C. elegans* Propagated with Stigmasterol and Either N,N-dimethyldodecanamide or N,N-dimethyldodecanamine[a]

	Dietary supplement[b]		
Recovered sterol	Stigmasterol	Stigmasterol + amide	Stigmasterol + amine
Stigmasterol	20.8	18.9	17.8
Cholesterol	9.1	10.9	5.7
7-Dehydrocholesterol	50.6	47.2	23.3
Lathosterol	4.8	7.3	4.4
Cholesta-5,7,9(11)-trienol	5.2	5.3	5.6
4α-Methylcholest-8(14)-enol	9.2	8.7	5.0
4α-Methylcholest-7-enol	0.3	0.5	1.7
Desmosterol	–	–	4.2
Cholesta-5,7,24-trienol	–	–	14.8
Cholesta-5,22E,24-trienol	–	–	3.0
Cholesta-5,7,22E,24-tetraenol	–	–	4.1
Cholesta-5,7,9(11),24-tetraenol	–	–	3.2
Cholesta-5,7,9(11),22E,24-pentaenol	–	–	0.6
4α-Methylcholesta-8(14),24-dienol	–	–	1.8
4α-Methylcholesta-7,24-dienol	–	–	0.9
Stigmasta-5,22E,24(28)-trienol	–	0.4	0.9
Other sterols (unidentified)	–	0.8	3.0
Total $\Delta^{22,24}$-sterols	–	–	7.7
Total of other Δ^{24}-sterols	–	–	24.9

[a]Dashed line (–) indicates sterol not detected.

[b]Media contained 25 ppm stigmasterol alone or in combination with 35 ppm amide or 25 ppm amine.

TABLE 2

Mass Spectral Data for Several Steryl Acetates Derived from *C. elegans* as Metabolites of Stigmasterol

	Cholesta-5,22E,24-trienyl acetate		Cholesta-5,7,22E,24-tetraenyl acetate		Cholesta-5,7,9(11),22E,24-pentaenyl acetate		Stigmasta-5,22E,24(28)-trienyl acetate	
Fragmentation[a]	(m/z)	(%)	(m/z)	(%)	(m/z)	(%)	(m/z)	(%)
M^+	424	1	422	1	420	1	452	1
M^+-Ac	364	1	362	26	360	14	392	12
M^+-Ac-CH_3	349	–	347	3	345	1	377	–
M^+-SC	315	–	313	–	311	–	315	–
M^+-SC-2H	313	8	311	–	309	–	313	18
M^+-Ac-(C-22 to C-29)	282	24	280	1	278	1	282	89
M^+-Ac-SC	255	6	253	10	251	14	255	24
M^+-Ac-SC-2H	253	16	251	6	249	7	253	78
M^+-Ac-SC-C_3H_6	213	2	211	2	209	6	213	8
M^+-Ac-SC-C_4H_8	199	1	197	3	195	7	199	4
$C_{12}H_{13}$	157	5	157	27	157	4	157	14
$C_{11}H_{11}$	143	6	143	22	143	1	143	14
SC	109	100	109	100	109	100	137	48
C_7H_{11}	95	18	95	8	95	3	95	100

[a]Ac = CH_3COOH; SC = side-chain.

Effects of N,N-Dimethyldodecanamide on Stigmasterol Metabolism

The addition of dimethyldodecanamide (35 ppm) to stigmasterol-supplemented (25 ppm) media inhibited population growth and reproduction of *C. elegans* as it did similarly when sitosterol was the dietary sterol (11). With stigmasterol, the amide caused a population decrease of 84% and a 71% reduction in the dry weight (after lyophilization) of harvested nematodes. No significant difference in sterol composition resulted (Table 1) except for the appearance of a relatively small percentage of a compound, tentatively identified as stigmasta-5,22E,24(28)-trienol, that was not detected in *C. elegans* fed stigmasterol alone. Its structural identity was suggested by the mass spectrum of its acetate (M^+, m/z 452), which included the following significant ions (Table 2): m/z 392, resulting from loss of acetic acid; 282, from additional cleavage at C-20(22); 255 and 253, from additional cleavage at C-17(20); 137, representing the side-chain moiety, and 95, possibly arising from the side-chain moiety after cleavage of the isopropyl group at C-24(25). Experimental RRT values for this tentatively-identified steryl acetate are included in Table 3. However, authentic stigmasta-5,22E,24(28)-trienol was unavailable.

Effects of N,N-Dimethyldodecanamine on Stigmasterol Metabolism

Dimethyldodecanamine (25 ppm) also inhibited reproduction of stigmasterol-supplemented (25 ppm) cultures as it did in previous studies with sitosterol-supplemented cultures (11). In the present case, the amine caused a 68% decrease in population and a 58% decrease in dry weight. However, unlike the amide, the amine appreciably altered the sterol composition of *C. elegans* (Table 1). Most notable were a large decrease in the relative percentage of 7-dehydrocholesterol and the presence of a number of sterols (33% of total sterol) possessing either a Δ^{24}- or a $\Delta^{22,24}$-side chain, none of which were detected in *C. elegans* fed stigmasterol alone (or with the inhibitory amide). Of these, cholesta-5,7,24-trienol was predominant. The Δ^{24}-sterols possessed properties which were identical to those of authentic standards and/or were in agreement with properties of com-

TABLE 3

GLC Analyses of Several Metabolites of Stigmasterol from *C. elegans*

	RRT[a]		
Steryl acetate	DB-1	SE-30	OV-17
Cholesta-5,7,9(11)-trienol	0.98	0.98	1.06
Cholesta-5,7,9(11),24-tetraenol	1.08	1.06	1.25
Cholesta-5,7,9(11),22E,24-pentaenol	1.17	1.12	1.48
Cholesta-5,22E,24-trienol	1.18	1.15	1.43
Cholesta-5,7,22E,24-tetraenol	1.30	1.25	1.68
Stigmasta-5,22E,24(28)-trienol	1.51	1.49	1.60

[a]Retention time of sterols as acetate derivatives relative to cholesteryl acetate. Experimental conditions are described in Materials and Methods section.

pounds previously isolated from *C. elegans*, e.g. cholesta-5,7,24-trienol (9-11).

Mass spectra of acetate derivatives of cholesta-5,22E,24-trienol and cholesta-5,7,22E,24-tetraenol from *C. elegans* (Table 2) matched those of authentic standards and agreed with previously-reported data for these sterols (21, 22). Both sterols from *C. elegans* exhibited two characteristics of a $\Delta^{22,24}$-conjugated diene system (21): each produced a mass spectral base ion at m/z 109 and demonstrated UV absorption (as acetates in hexane) at λ_{max} 229 sh, 236, 245 sh nm. The tetraenyl acetate showed additional absorption peaks at λ_{max} 260 sh, 269, 280, 291 nm, typical of a $\Delta^{5,7}$-diene system (23). The acetates of cholesta-5, 22E,24-trienol and cholesta-5,7,22E,24-tetraenol from *C. elegans* possessed RRT's (Table 3) identical to those of standards.

The assignment of cholesta-5,7,9(11),22E,24-pentaenol (for which no authentic standard was available) was corroborated by the mass spectrum of its acetate (Table 2): peaks at m/z 420 (M^+), 360 and 251 indicated a cholestanol acetate structure with five double bonds; the base peak at m/z 109 indicated a $\Delta^{22,24}$-side chain, and significant peaks at m/z 209 and 195 suggested a $\Delta^{5,7,9(11)}$-nucleus (10,12). A UV spectrum of the pentaenyl acetate revealed characteristic absorption for both a $\Delta^{22,24}$-diene and a $\Delta^{5,7,9(11)}$-triene system, the latter occurring at λ_{max} 306, 322, 338 nm (23). The expermental RRT's for the pentaenyl acetate (Table 3) were in close agreement with calculated values based on GLC separation factors for $\Delta^{5,7,9(11)}$- and $\Delta^{22,24}$-bonds, factors derived from the RRT's of authentic standards of cholesta-5,7, 9(11)-trienol and cholesta-5,22E,24-trienol (Table 3). Tentatively-identified stigmasta-5, 22E,24(28)-trienol also was detected in the presence of the amine. During argentation column chromatography, the acetate derivative of stigmasta-5,22E,24(28)-trienol was eluted after cholesta-5,7,9(11)-trienyl acetate but prior to cholesta-5,22E-24-trienyl acetate.

When [^{3}H] stigmasterol was utilized in media containing either the amine or amide, all sterols from *C. elegans* were radiolabeled with approximately the same specific activity as the dietary stigmasterol, as determined by radioassay of trapped GLC effluent. Thus, all sterols isolated from *C. elegans* originated from the dietary stigmasterol, confirming our earlier conclusion that *C. elegans* is capable of dealkylating stigmasterol (12).

Metabolism of Campesterol

C. elegans, when propagated in media con-

TABLE 4

Relative Percentages of Total Sterols from *C. elegans* Propagated with Different Dietary Sterols[a,b]

	Dietary supplement			
	Campesterol	Campesterol + amine[c] (10 ppm)	Campesterol + amine[c] (25 ppm)	24-Methylene-cholesterol
Cholesterol	4.0	2.6	0.8	9.3
7-Dehydrocholesterol	26.3	26.8	12.5	47.7
Lathosterol	3.4	2.1	0.7	4.3
Cholesta-5,7,9(11)-trienol	1.5	1.6	0.8	2.2
Campesterol	38.9	37.0	60.0	–
Campesta-5,7-dienol	13.9	14.2	12.5	–
Campest-7-enol	0.7	0.7	–	–
Campesta-5,7,9(11)-trienol	0.9	1.3	0.6	–
24-Methylenecholesterol	3.2	3.8	1.9	27.3
Campesta-5,7,24(28)-trienol	1.1	1.0	0.2	3.4
4α-Methylcholest-8(14)-enol	4.6	4.3	1.6	4.3
4α-Methylcholest-7-enol	0.2	0.8	0.9	0.3
4α,24-Dimethylcholest-8(14)-enol	0.8	0.9	0.4	–
4α,24-Dimethylcholest-7-enol	–	0.2	0.3	–
4α,24-Dimethylcholestanol	0.5	0.6	0.3	–
Cholesta-5,7,24-trienol	–	1.7	5.4	0.1
4α-Methylcholesta-8(14),24-dienol	–	0.3	0.4	–
4α-Methylcholesta-7,24-dienol	–	0.1	0.3	–
Other sterols (unidentified)	–	–	0.4	1.1
Total Δ^{24}-sterols	–	2.1	6.1	–

[a]Media were supplemented with 25 ppm sterol.

[b]Dashed line (–) indicates sterol not detected.

[c]N,N-dimethyldodecanamine.

taining campesterol alone, gave results that were in agreement with our previous study (12). As shown in Table 4, *C. elegans* fed only campesterol contained, excluding dietary sterol, mostly 7-dehydrocholesterol and campesta-5,7-dienol with smaller percentages of various other sterols whose identities have been discussed previously (12).

Effects of N,N-Dimethyldodecanamine on Campesterol Metabolism

Two different concentrations of dimethyldodecanamine were used to supplement the campesterol media of *C. elegans*. A 10 ppm concentration of the amine resulted in a 16% reduction in the nematode population and a 13% reduction in dry weight. The sterol composition of this amine-treated (10 ppm) culture (Table 4) was virtually unchanged from that of the non-treated culture except for the presence of a small percentage (2% of total sterol) of several Δ^{24}-sterols in amine-treated *C. elegans*.

Treatment with a higher amine concentration, 25 ppm, decreased the population by 72% and reduced the dry weight by 82%. Treatment of *C. elegans* at this concentration substantially altered its sterol composition (Table 4), producing a greater percentage of Δ^{24}-sterols (6%), primarily cholesta-5,7,24-trienol, than did the lower amine concentration. Other substantial differences were a decreased percentage of 7-dehydrocholesterol and an increased proportion of campesterol upon treatment with 25 ppm amine.

The variety of 4-methylsterols which were detected provided special interest owing to their novel biosynthesis in *C. elegans* (9). Therefore, some of their analytical properties are presented. GLC data for the recovered 4-methylsterols are listed in Table 5. Their RRT's as acetates were about 3% less than their RRT's as free alcohols, a phenomenon characteristic of 4-methylsterols (24) and not exhibited by the other compounds identified here as 4-desmethylsterols. The 4-methylsterol RRT's were in close agreement with standard reference values given elsewhere (25). Mass spectral data for the acetates of 4α,24-dimethylcholest-8(14)-enol and 4α,24-dimethylcholest-7-enol are presented and compare favorably with their 4-methylsterol counterparts (Table 6). Fragment ions from the 4α,24-dimethylsterols with intact side-chains were 14 m/z units greater than their corresponding 4α-methylsterol fragments. M^+-Ac-SC peaks (m/z 269) were much more intense for the Δ^7-sterols than for the $\Delta^{8(14)}$-sterols.

Metabolism of 24-Methylenecholesterol

C. elegans propagated with 24-methylenecholesterol grew very well (ca 67,000 nematodes/ml; dry wt, 1.82 g/l of media), as it did similarly when propagated with campesterol, stigmasterol or sitosterol (12). Nematodes fed 24-methylenecholesterol contained predominantly 7-dehydrocholesterol and smaller amounts of various other 24-desalkylsterols (Table 4). *C. elegans* is thus capable of dealkylating a 24-alkylsterol which contains a $\Delta^{24(28)}$-bond. 24-Methylenecholesterol was dealkylated to a greater extent than campesterol, as dietary 24-methylenecholesterol yielded greater percentages of 7-dehydrocholesterol and cholesterol and a smaller percentage of unmetabolized dietary sterol. Furthermore, dietary campesterol produced a relatively large percentage (21%) of various metabolites with a 24-methyl group (mostly campesta-5,7-dienol), whereas dietary 24-methylenecholesterol yielded only one metabolite with a 24-methylene group, campesta-5,7,24(28)-trienol, comprising a small percentage of the total sterol. These observa-

TABLE 5

GLC Analyses of 4-Methylsterols from *C. elegans* Propagated with Campesterol

	RRT[a]			
	OV-17		DB-1	
	Free sterol	Acetate	Free	Acetate
4α-Methylcholest-8(14)-enol	1.16	1.13	1.17	1.13
4α-Methylcholest-7-enol	1.36	1.32	1.30	1.27
4α-Methylcholesta-8(14),24-dienol	1.40	1.37	1.28	1.24
4α-Methylcholesta-7,24-dienol	1.63	1.57	1.44	1.40
4α,24-Dimethylcholest-8(14)-enol	1.52	1.48	1.50	1.46
4α,24-Dimethylcholestanol	1.52	1.48	1.53	1.50
4α,24-Dimethylcholest-7-enol	1.80	1.76	1.67	1.62

[a]RRT = retention time of free sterol relative to cholesterol; retention time of steryl acetate relative to cholesteryl acetate. Experimental conditions are described under Materials and Methods.

TABLE 6

Mass Spectral Data for 4-Methylsteryl Acetates Derived from *C. elegans* Propagated with Campesterol

Fragmentation[a]	4α-Methylcholestenyl acetate			4α-24-Dimethylcholestenyl acetate		
	(m/z)	$\Delta^{8(14)}$ (%)	Δ^{7} (%)	(m/z)	$\Delta^{8(14)}$ (%)	Δ^{7} (%)
M^+	442	36	33	456	20	26
M^+-CH_3	427	4	4	441	2	2
M^+-Ac	382	2	4	396	1	2
M^+-Ac-CH_3	367	7	6	381	2	3
M^+-SC	329	4	4	329	3	4
M^+-Ac-SC	269	14	52	269	9	53
M^+-Ac-SC-C_2H_2	243	23	18	243	15	14
M^+-Ac-SC-C_3H_6	227	34	36	227	23	33
C_7H_{11}	95	73	96	95	64	93
C_4H_7	55	100	100	55	100	100

[a]Ac = CH_3COOH; SC = side-chain.

tions demonstrate a more efficient dealkylation and conversion of 24-methylenecholesterol than campesterol, thus supporting the proposal of 24-methylenecholesterol as an intermediate in the pathway of campesterol dealkylation in *C. elegans* (12).

DISCUSSION

In the absence of an inhibitor, stigmasterol and sitosterol were metabolized similarly in *C. elegans* (10,12). Even the effects of either of the two inhibitors on the metabolism of these two sterols were similar. Previously (11), sitosterol metabolism was unaffected by treatment of *C. elegans* with the amide except for a small percentage (1.1% of total sterol) of fucosterol detected in the presence of the amide. Similarly, in the present study, stigmasterol metabolism was unchanged by the presence of the amide except for the detection of a small percentage (0.4%) of tentatively-identified stigmasta-5,22E,24(28)-trienol (i.e., either 22-dehydrofucosterol or 22-dehydroisofucosterol) which also was detected in the presence of the amine. The conversion of sitosterol to fucosterol is believed to be the initial step in the C-24 dealkylation process in *C. elegans* (10); it has been demonstrated as the initial step in sitosterol dealkylation by the tobacco hornworm, *Manduca sexta* (L.) (26), and the silkworm, *Bombyx mori* (27). An analogous comparison suggests that stigmasta-5,22E, 24(28)-trienol, possibly the 22-dehydrofucosterol isomer, is the first intermediate in stigmasterol dealkylation by *C. elegans* (Fig. 1). Fujimoto et al. (17) have demonstrated recently that the 24(28)E- and Z-isomers of stigmasta-5, 22E,24(28)-trienol were both dealkylated by silkworm larvae to produce cholesta-5,22,24-trienol, desmosterol and cholesterol.

The next step in the dealkylation process, at least in insects, is believed to be the conversion of fucosterol and 22-dehydrofucosterol to their respective 24(28)-epoxides (17,27). The amide apparently exhibits slight inhibition of the 24(28)-epoxidase, perhaps due to the amide's slight steric resemblance to the ethylidene side-chain of fucosterol or 22-dehydrofucosterol. However, inhibition of the Δ^{24}-sterol reductase by the amide does not occur. Loss of the amide's carbonyl group results in inhibition of the reductase, as evidenced by the activity of the amine which caused the accumulation of a large percentage of Δ^{24}-sterols. The carbonyl group may sterically hinder binding between the amide and the enzyme. Alternatively, the basicity of the amine molecule may be responsible for its binding to the enzyme, and such basicity is eliminated when the carbonyl group of the amide is present.

Of particular interest was the appearance of the $\Delta^{22,24}$-sterols in amine-treated nematodes. The tobacco hornworm had provided the first isolation of such sterols from a biological source (28). The existence of the $\Delta^{22,24}$-sterols in *C. elegans*, e.g., cholesta-5,22E,24-trienol, indicates that removal of the C-24 ethyl substituent of stigmasterol can proceed in the presence of the Δ^{22}-bond. The presence of Δ^{24}-sterols, e.g., desmosterol, and the failure to detect any Δ^{22}-sterols, e.g., 22-dehydrocholesterol, demonstrates that, with a $\Delta^{22,24}$-sterol substrate, Δ^{22}-reduction occurred prior to Δ^{24}-reduction. Thus, the sequence of stigmasterol dealkylation in *C. elegans* is postulated in Figure 1. The existence of an alternative sequence of $\Delta^{22,24}$-reduction (cholesta-5,22,24-trienol to 22-dehydrocholesterol to cholesterol) cannot be ruled out. If this sequence also occurred in *C. elegans*,

inhibition of the Δ^{24}-sterol reductase still would have resulted in accumulation of $\Delta^{22,24}$-sterols. However, unless the amine also inhibited the Δ^{22}-sterol reductase, Δ^{22}-sterols (22-dehydrocholesterol or cholesta-5,7,22-trienol) would not have accumulated but would have been reduced further, accounting for the absence of Δ^{22}-sterols.

Metabolism of stigmasterol by *C. elegans* appears to be quite similar to that process in the silkworm (17), the tobacco hornworm (28) and *Tribolium confusum*, the confused flour beetle (22): C-24 dealkylation produces a $\Delta^{22,24}$-sterol whereupon the Δ^{22}-bond is reduced before the Δ^{24}-bond. *C. elegans* shares another similarity with *T. confusum*: their ability to introduce a Δ^{7}-bond and consequently produce 7-dehydrocholesterol as the predominant metabolite of most phytosterols. Cholesta-5,7,24-trienol and cholesta-5,7,22E,24-tetraenol, both detected in *C. elegans* fed stigmasterol and the amine, were likewise found in *T. confusum* fed stigmasterol and 22,25-diazacholesterol, another Δ^{24}-sterol reductase inhibitor (22). An interesting aspect of stigmasterol metabolism in the hornworm is the apparent requirement of the Δ^{24}-bond in a $\Delta^{22,24}$-sterol substrate in order for reduction of the Δ^{22} bond to occur efficiently. Dietary 22-dehydrocholesterol was not converted to cholesterol by the hornworm (29). A similar substrate specificity of the Δ^{22}-reductase in *C. elegans* might also exist.

Inhibition of the Δ^{24}-sterol reductase by the amine was also observed in campesterol-supplemented *C. elegans* as Δ^{24}-sterols comprised 6% of the total sterol. However, at the same amine concentration (25 ppm), reductase inhibition appeared to be much greater in cultures supplemented with sitosterol (11) or stigmasterol where Δ^{24}-sterols comprised 27% and 33%, respectively, of the total sterol. Also in contrast, 25 ppm amine caused a large accumulation of unmetabolized dietary campesterol but did not change the relative percentage of unmetabolized dietary sterol when either sitosterol (11) or stigmasterol was utilized. The increased proportion of campesterol may have been due to a greater uptake of that dietary sterol from the media as a result of some interaction between the amine and campesterol upon the nematodes. The sterol content of *C. elegans* propagated with campesterol was 1.21 mg/g dry wt, similar to that obtained from sitosterol- or stigmasterol-propagated cultures. *C. elegans* in media containing campesterol and 25 ppm amine yielded 1.61 mg sterol/g dry wt, whereas similar increases were not observed in amine-treated *C. elegans* supplemented with either sitosterol or stigmasterol. Although the population decrease due to the amine was comparably 70% with both dietary sterols, there was a considerable difference in the decrease in dry weight (60% with stigmasterol, and 80% with campesterol). The difference could be due to a greater percentage of larger nematodes (perhaps adults) in amine-treated, stigmasterol-supplemented cultures or, conversely, a greater percentage of smaller nematodes in amine-treated, campesterol-supplemented cultures. The higher uptake of campesterol may have repressed the development and size of individual nematodes. Another possible reason for the increased proportion of campesterol may be the ability of the amine to inhibit an additional enzymatic site in *C. elegans*: the conversion of campesterol to the first intermediate, presumably 24-methylenecholesterol, via a C-24(28)-dehydrogenase. The amine does not, however, inhibit the corresponding conversions of sitosterol and stigmasterol to fucosterol and 22-dehydrofucosterol, respectively, suggesting a C-24(28)-dehydrogenase system in *C. elegans* which exhibits specificity toward the size (methyl vs. ethyl) of the 24-alkyl substituent of the sterol substrate. Figure 2 illustrates the sites of sterol metabolism in *C. elegans* inhibited by the amine.

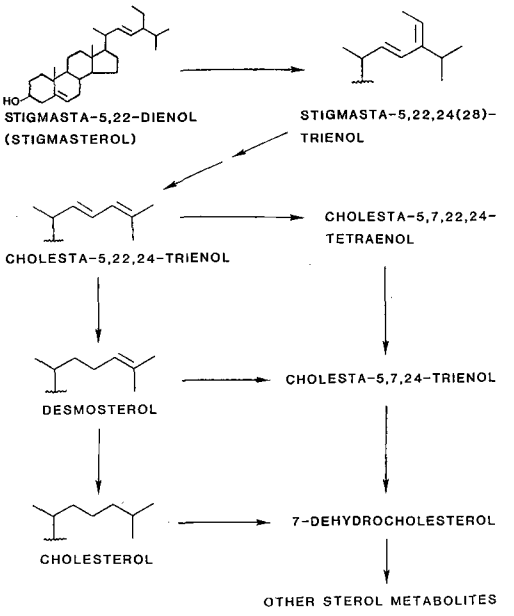

FIG. 1. Proposed metabolism of stigmasterol by *C. elegans*.

The C-24 demethylation process in *C. elegans* is perhaps similar to those in *B. mori* (15), *Tenebrio molitor* (16) and *M. sexta* (30), which proceed via 24-methylenecholesterol as the initial intermediate between campesterol and cholesterol. However, whereas desmosterol was identified in *B. mori* (15) and *M. sexta* (31) as

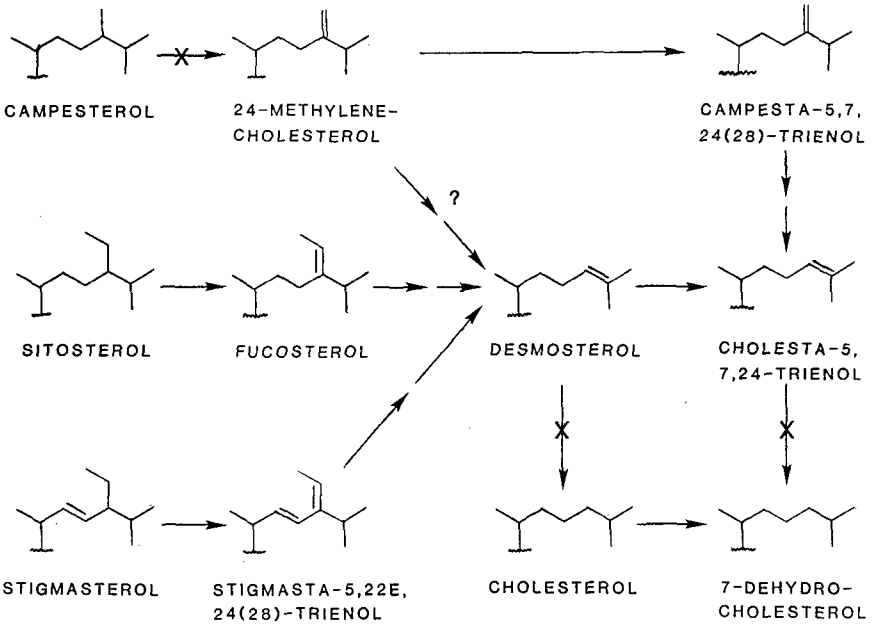

FIG. 2. Sites (marked by "x") of sterol metabolism in *C. elegans* inhibited by N,N-dimethyldodecanamine. Question mark indicates failure to detect desmosterol in *C. elegans* fed campesterol or 24-methylenecholesterol.

an intermediate from both campesterol and 24-methylenecholesterol to cholesterol, we failed to detect desmosterol as a metabolite of these sterol substrates in *C. elegans*, even in the presence of the Δ^{24}-sterol reductase-inhibiting amine. Nevertheless, we did detect various other Δ^{24}-sterols, and desmosterol remains as a probable intermediate from campesterol in *C. elegans*. Failure of the amine to cause accumulation of desmosterol may have been a result of its primary inhibition of the campesterol C-24(28)-dehydrogenase (Fig. 2). The intermediacy of a $\Delta^{24(28)}$-sterol in the dealkylation of 24-alkylsterols is not a mechanism shared by all organisms that are capable of C-24 dealkylation. Unlike *C. elegans* and the above-mentioned insects, the protozoan *Tetrahymena pyriformis* cannot dealkylate either 24-methylsterols or $\Delta^{24(28)}$-sterols (24-methylenecholesterol, fucosterol or isofucosterol), although it is capable of dealkylating 24-ethylsterols (32).

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Influence of Dietary Cholesterol on the Relative Synthesis of Hepatic Glycerides and Molecular Classes of 1,2-Diglycerides and Phospholipids in the Gerbil in vivo

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ABSTRACT

The influence of dietary cholesterol on the relative rates of synthesis of hepatic lipids in the male Mongolian gerbil, *Meriones unguiculatus*, was studied. The semi-purified starch-based diet used lard as the dietary fat and was fed with or without a 0.5% (by wt.) cholesterol supplement. Each animal received 300 μCi [2-^{3}H]-glycerol i.p. after 3 or 7 days on the dietary regimens. Relative rates of [2-^{3}H]-glycerol incorporation into the major hepatic glycerides in vivo was not affected significantly by dietary cholesterol (0.5% level), suggesting that alteration in the relative biosynthesis of these lipids could not readily account for the higher triglyceride (TG) to phospholipid (PL) mass ratio in liver with cholesterol feeding. However, there was evidence for an increased formation of 1,2-diglyceride (1,2-DG). The complement of molecular species of hepatic 1,2-DG, phosphatidylcholine (PC), and phosphatidylethanolamine (PE) formed de novo, as measured using isotopic glycerol, was not influenced greatly by dietary cholesterol, although lower mean rates of synthesis of tetraenoic relative to dienoic species of phospholipids were indicated in cholesterol-fed gerbils.

Lipids 20:167-172.

INTRODUCTION

It has been demonstrated in various animal species that the supplementation of diets with large amounts of cholesterol can affect the level and composition of hepatic glycerides (1-4). In the Mongolian gerbil, *Meriones unguiculatus*, semi-synthetic diets supplemented with cholesterol at 0.1 or 0.5% by wt. produced elevated hepatic TG and 1,2-DG levels but had little effect on hepatic total PL levels (5). The mass ratio of TG to PL in liver was elevated in gerbils and rats fed cholesterol-supplemented diets (1,5).

The arachidonate (20:4) to linoleate (18:2) fatty acid ratio is reduced in tissue lipids of different animal species by dietary cholesterol, and it has been suggested that the % conversion of linoleate to arachidonate may be suppressed by dietary cholesterol (1,2,5,6). It also has been suggested that dietary cholesterol may impair the metabolic conversion of linolenate to docosahexaenoate (22:6) as supported by reduced levels of this latter acid in the hepatic phospholipids of gerbils receiving a cholesterol supplement. Changes in the 18:0 to 16:0 fatty acid ratios and in the 18:1 to 18:0 ratios as a function of cholesterol feeding were observed in gerbil liver 1,2-DG as well as PL and/or TG. Cholesterol supplementation (0.5% by wt.) produced a lower 18:0/16:0 ratio in 1,2-DG but had no effect on this ratio in liver TG; on the other hand, the 18:0/16:0 ratio was elevated in total PL and in PC. The 18:1/18:0 ratio was higher in hepatic total PL, PE, 1,2-DG and TG with dietary cholesterol supplementation. In the rat, as well, the 18:1/18:0 ratio was elevated with cholesterol feeding (1).

It also has been suggested that dietary cholesterol might affect the synthesis of fatty acids utilized for incorporation into mitochondrial PC and PE in liver differentially (8). The % of 20:4 was depressed more in PC than in PE by dietary cholesterol; the % of 16:1 was elevated in PC but depressed in PE as a function of the cholesterol supplement. In gerbil liver, the percentages of various fatty acids in PC and PE appeared to respond differentially to cholesterol feeding for 3 to 7 days (5).

Various possible explanations for the influence of dietary cholesterol on TG, DG and PL levels include dietary cholesterol mediation of lipid synthesis, lipolysis, or mobilization from liver via lipoprotein assembly and release into the circulation. It was thus desirable to determine whether the altered mass of certain hepatic lipids may be due to different relative rates of synthesis of these lipids. Furthermore, the types of molecular species of 1,2-DG, PC, and PE that are formed de novo with and without dietary cholesterol supplementation also were examined.

MATERIALS AND METHODS

Upon receipt, 32 young (ca. 2 mo of age) male gerbils (High Oak Ranch, Goodwood, Ontario) were randomized into 4 groups of 8 animals each, housed 2 per screen-bottomed

cage in a diurnally-lighted room with water ad lib, and fed either a semi-purified starch-based basal diet or the basal diet supplemented with 0.5% cholesterol (9). Two groups received the different diets for 3 days, and the other 2 groups received the different diets for 7 days. After a 4-hr fast each animal was injected i.p. with 0.3 mCi [2-^{3}H]-glycerol (specific activity 200 mCi/mmol) in sterile aqueous solution. Animals were killed 10 min post-injection. This time period was chosen with regard to previous time-study investigations which showed the 10-min mark to be midway in the period of rapid, steady synthesis of TG and the various PL. Figure 1 shows the results of the time-study with 12 gerbils. Each point represents the average of 2 young male gerbils, which had been maintained, then fasted, as described above using a commercial chow as the pelleted diet (Lab Chow No. 5001, Ralston-Purina Co., St. Louis, Missouri). Livers were rapidly excised and homogenized in 20 volumes of chloroform-methanol (2:1, v/v). A water wash was used. Aliquots of the chloroform phases were taken for lipid fractionation by thin-layer chromatography (TLC) to determine first the relative incorporation of tritium label into the various hepatic lipid fractions and, second, the relative incorporation of tritium label into the molecular species of hepatic 1,2-DG, PC, and PE.

The "neutral lipid plate" (by TLC) was used to isolate total PL, 1,2-DG, 1,3-DG, and TG; the "Skipski plate" was used to isolate PC, PE, phosphatidylserine (PS), and phosphatidylinositol (PI) (10). Gel scrapings were dispersed in 1.5 ml water to which 13.5 ml Aquasol-2 (New England Nuclear Corp., Boston, Massachusetts) were added for liquid scintillation counting. Tritium dpm were calculated by means of blank subtraction and a tritium quench correction curve using the external standard ratio method (11), and results were expressed as dpm per gram liver.

Of the animals injected with tritiated glycerol, the distribution of label among molecular species of liver 1,2-DG, PC, and PE was determined from cholesterol-supplemented and cholesterol-unsupplemented animals fed 7 days. The 1,2-DG, PC, and PE were isolated as previously described. Bands were scraped into screw-top test tubes and 6.0 ml aliquots of elution solvent (chloroform/methanol/acetic acid/water, 50:39:1:10, v/v/v/v) were added. After vortexing and a short centrifugation (ca. 5 min 1000Xg), supernatants were decanted into new test tubes, and half-volumes of eluant were added to the sediment for reextraction. To each of the total supernatants, 1/3 vol. of 4M ammonia was added; after vortexing, lower phases were transferred to new test tubes to which water/methanol (1:1, v/v) was added with mixing in an amount equal to the volume of

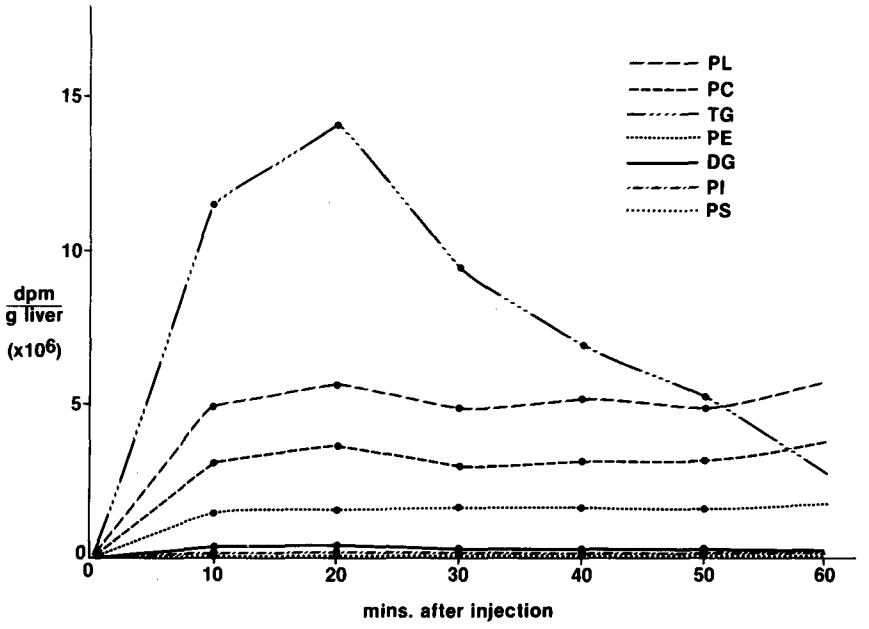

FIG. 1. The incorporation of radioactivity into various hepatic lipids after injection of [2-^{3}H]-glycerol.

ammonia previously added. Solutions were spun (1000Xg) to achieve a clear phase separation.

Phospholipase C from Type V *B. cereus* (EC No. 3.1.4.3.) (Sigma Chemical Co., St. Louis, Missouri) was used to obtain DG from PC and PE. For PC, a volume of the previously prepared chloroform phase was evaporated; 2 ml of 50 mM Tris buffer (pH 7.4) were added with a 30 sec shake; then 2 ml diethyl ether were added with a 30 sec shake; then 5 units of phospholipase C were added with a 30 min shake. The ether layer was transferred to a new test tube; the remaining mix was washed with another 2 ml of diethyl ether. Ether phases were combined and evaporated; 30 μl chloroform/methanol (2:1, v/v) were added, and the DG was isolated by TLC on silica gel G in heptane/isopropyl ether/acetic acid. The DG was eluted from the gel with diethyl ether/methanol/acetic acid (60:40:1, v/v/v). Fifty μl chloroform/methanol (2:1, v/v) were added to the dried DG, and DG molecular species were separated by argentation TLC on silica gel G (impregnated 15% by wt. with silver nitrate) in chloroform/diethyl ether (95:5, v/v). The DG molecular species of PE were similarly obtained except for the following differences: PE was incubated with 5 units of phospholipase C for 60 min; exogenous cold, 1,2-DG was added as a carrier for visualizing the DG molecular species from argentation TLC. The molecular species of the gerbil liver free 1,2-DG also were separated with the addition of exogenous cold 1,2-DG carrier. After TLC plates had dried, bands of the molecular species of the PC, PE and 1,2-DG were scraped into scintillation vials to which 15 ml of OCS fluid (Amersham Corp., Oakville, Ontario) were added. Saturates and monoenes were combined; other bands were dienes, trienes, tetraenes, pentaenes and hexaenes. Tritium was counted in a Searle Delta 300 counter, using the single window module.

Statistical analyses were conducted with the use of Student's t-test (12). The significance level was chosen to be $p<0.05$.

RESULTS

Feeding dietary cholesterol for 7 days did not influence food intake (each gerbil consumed about 4 g per d), weight gain (1.0 ± 0.1 g per d as mean ±SE for all 7-day animals), or liver weight (1.6 ± 0.1 g as mean liver weight for all 7-day animals and 4.1 ± 0.2 g per 100 g body weight as mean relative liver weight of all 7-day animals, n=16) (5,9). The incorporation of [2-^{3}H]-glycerol into liver TG, DG, PL, PC, PE, PI and PS is given as a function of post-injection time in Figure 1. The amount of radioactivity accumulating in the various lipids approached a maximum at 20 min after which it fluctuated moderately in all lipids with the exception of TG, where it fell steadily until 60 min post-injection. Based on these results, an exposure time of 10 min to [^{3}H]-glycerol was used routinely in all other experiments since it most likely would provide information on the relative rates of lipid synthesis in animals from the different dietary treatments.

The extent of uptake of [^{3}H]-glycerol into the livers, total lipids, or total water-soluble metabolites was not influenced by dietary cholesterol after 3 or 7 days on the experimental diets (Table 1). The entry of labeled glycerol into hepatic TG remained steady at days 3 and 7, whereas the incorporation into PL was less at day 7 regardless of dietary regime. The amount of radioactivity in the 1,2-DG was significantly higher and that in the PS lower in animals fed the cholesterol-supplemented diet for 7 days relative to controls. No differences were found in the other lipid classes at day 7.

The percentage distribution of radioactive label among the various lipid fractions as a function of dietary treatment is given in Table 2. The proportion of radioactivity found in the 1,2-DG fraction was significantly higher in animals receiving 0.5% cholesterol at both days 3 and 7. No statistically significant difference between dietary treatments was found among other lipid classes, although the mean percentage in PS was lower by 20-25% for animals fed the cholesterol supplement. The relative rates of isotope incorporation into TG versus PL, TG versus PC, or TG versus PE were not affected by dietary cholesterol supplementation, although the PC to PE ratio was enhanced by dietary cholesterol after 3 but not after 7 days (Table 2). The TG/PL count ratio was higher in the 7-day versus the 3-day gerbils regardless of dietary treatment.

Table 3 gives the percentage distribution of radioactive glycerol among the various molecular species of free 1,2-DG, PC and PE after isotope administration. The majority of the radioactivity associated with the free 1,2-DG and PC was found in the oligoenoic (saturated plus monoenoic plus dienoic) species with very little in the tetraenes (3% or less), pentaenes (<3%), or hexaenes (3% or less). In contrast, the PE contained considerable radioactivity in the pentaenes (10-12%) and hexaenes (33-34%) as well as the oligoenes (49-52%), with little in the tetraenes (<4%). Dietary cholesterol was essentially without effect on the distribution of [^{3}H]-glycerol among the various molecular classes of 1,2-DG, PC or PE, although a moder-

TABLE 1

Effect of Dietary Cholesterol Level on the Distribution of Radioactivity Among Liver Lipids 10 Min Post-Injection of [2-^{3}H]-Glycerol (10^3 dpm per g liver ± SE)

	Dietary treatment			
	3-day trial		7-day trial	
Lipid fraction	Starch	+0.5% Cholesterol	Starch	+0.5% Cholesterol
Triglyceride	10554 ± 839	9990 ± 560	10140 ± 1442	9740 ± 1012
1,2-Diglyceride	329 ± 12	377 ± 39	221 ± 23	413 ± 66*
1,3-Diglyceride	129 ± 11	83 ± 10*	104 ± 18	88 ± 16
Total phospholipid	5937 ± 639	5173 ± 423	3892 ± 416	3678 ± 234
Phosphatidylcholine	4245 ± 447	4037 ± 381	2762 ± 306	2558 ± 191
Phosphatidylethanolamine	1688 ± 197	1345 ± 120	1178 ± 126	1060 ± 76
Phosphatidylserine	55 ± 8	42 ± 7	36 ± 4	27 ± 2*
Phosphatidylinositol	95 ± 20	90 ± 9	69 ± 10	69 ± 7
Total lipid phase	19454 ± 1510	17996 ± 1178	16283 ± 2017	15858 ± 1307
Total aqueous phase	2987 ± 881	1820 ± 140	8094 ± 4323	5253 ± 2214
Total	22440 ± 1797	19816 ± 1270	24377 ± 5242	21111 ± 3083

Values are the means (±SE) of 8 animals per group, except the diglycerides for which each value is the mean (±SE) of 4 paired samples per group.

*Denotes a significant difference by t-test from the corresponding cholesterol-unsupplemented group.

TABLE 2

Effect of Dietary Cholesterol Level on the Percentage Distribution of Radioactivity in Liver Lipid and on the Relative Rates of Synthesis of Certain Liver Lipids

	Dietary treatment			
	3-day trial		7-day trial	
Lipid fraction	Starch	+0.5% Cholesterol	Starch	+0.5% Cholesterol
	(%±SE)			
Triglyceride	62.1 ± 2.5	63.8 ± 0.8	69.4 ± 1.3	68.8 ± 1.9
1,2-Diglyceride	1.86 ± 0.12	2.42 ± 0.16*	1.56 ± 0.18	2.94 ± 0.28*
Total phospholipid	34.5 ± 2.3	32.7 ± 0.7	27.6 ± 1.3	27.0 ± 2.0
Phosphatidylcholine	24.2 ± 1.5	23.9 ± 0.9	18.9 ± 1.2	17.9 ± 1.4
Phosphatidylethanolamine	9.62 ± 0.75	8.01 ± 0.23	8.05 ± 0.49	7.34 ± 1.4
Phosphatidylserine	0.32 ± 0.04	0.24 ± 0.03	0.25 ± 0.02	0.20 ± 0.03
Phosphatidylinositol	0.53 ± 0.10	0.54 ± 0.04	0.46 ± 0.04	0.48 ± 0.04
		(relative rate ± SE)		
TG/PL	1.9 ± 0.2	2.0 ± 0.1	2.6 ± 0.2	2.7 ± 0.2
TG/PC	2.6 ± 0.2	2.6 ± 0.1	3.7 ± 0.3	3.9 ± 0.4
TG/PE	6.7 ± 0.6	7.6 ± 0.4	8.5 ± 0.6	9.3 ± 0.8
PC/PE	2.5 ± 0.1	3.0 ± 0.1*	2.4 ± 0.1	2.4 ± 0.1

Values are the means (±SE) of 8 animals per group, except the 1,2-DG for which each value is the mean (±SE) of 4 paired sampled per group.

*Denotes a significant difference by t-test from the corresponding cholesterol-unsupplemented group.

TABLE 3

Effect of Dietary Cholesterol Level on the Percentage Distribution of Radioactivity Among Molecular Species of 1,2-Diglyceride, Phosphatidylcholine and Phosphatidylethanolamine of Gerbil Liver from Animals Fed 7 Days (% ± SE)

	1,2-diglyceride		Phosphatidylcholine		Phosphatidyl ethanolamine	
Molecular species	Starch	+0.5% Chol.	Starch	+0.5% Chol.	Starch	+0.5% Chol.
Saturates plus monoenes	47.0 ± 3.4	47.3 ± 3.8	42.8 ± 2.0	42.1 ± 1.8	22.1 ± 2.2	22.1 ± 2.3
Dienes	38.0 ± 1.2	38.0 ± 1.0	48.1 ± 1.5	49.5 ± 2.0	26.4 ± 1.4	29.5 ± 1.8
Trienes	10.0 ± 1.6	9.7 ± 2.1	1.3 ± 0.1	1.5 ± 0.2	1.9 ± 0.2	2.6 ± 0.2*
Tetraenes	1.7 ± 0.3	2.4 ± 1.0	3.0 ± 0.3	2.1 ± 0.2[n]	3.8 ± 0.3	3.0 ± 0.3[n]
Pentaenes	1.2 ± 0.3	1.2 ± 0.3	2.3 ± 0.1	1.8 ± 0.3	11.9 ± 1.1	10.1 ± 0.8
Hexaenes	2.1 ± 0.3	1.6 ± 0.3	2.6 ± 0.3	3.0 ± 0.4	33.9 ± 2.5	32.7 ± 2.9

Values are the means (±SE) of 4 paired samples per group.

*Denotes a significant difference by *t*-test ($p<0.05$) from the corresponding cholesterol-unsupplemented group.

[n]Denotes that the p value for tetraene percentages in PC is 0.08 and for tetraene percentages in PE is 0.12.

Mean (±SE) values of the tetraene/diene ratios in PC and PE are 0.06 ± 0.01 versus 0.04 ± 0.004 and 0.14 ± 0.01 versus 0.11 ± 0.02 for basal versus cholesterol-supplemented groups, respectively. The p values for the tetraene/diene ratios in PC and PE are 0.07 and 0.12, respectively.

ately greater percentage of radioactivity was found in the trienoic species of PE with cholesterol supplementation. A general lowering in the tetraenoic/dienoic biosynthetic ratio in the case of PC and PE was associated with the ingestion of 0.5% cholesterol.

DISCUSSION

Glycerol-[2-^{3}H] was used as a lipid precursor in these experiments because its initial entry into glycerolipid via known metabolic pathways (13) represents de novo synthesis of lipid (14). This radioactive compound enters into lipid via the α-glycerophosphate pathway of acylation to phosphatidic acid and subsequently to 1,2-DG (14). The decay of radioactivity from TG in the time study after 20 min likely represents the mobilization of TG from liver as lipoprotein. Such a decay of radioactivity from the hepatic TG has been observed in isotopic studies using labelled fatty acid in rats (15). The results in Tables 1 and 2 suggest that there is an increased formation of 1,2-DG with cholesterol feeding, which is consistent with the elevated level of 1,2-DG in the livers of these gerbils (5). It is less likely that the elevation in 1,2-DG levels is due to an impaired utilization of this intermediate, because total glyceride levels in both liver and plasma are elevated with cholesterol feeding (5).

The relative rates of utilization of the intermediary 1,2-DG for TG and PL synthesis were not influenced by dietary cholesterol in a statistically significant manner as evidenced by the ratios in Table 2, thereby not apparently accounting for the higher TG/PL mass ratio found in liver with cholesterol feeding (5)However, the small but statistically insignificant increase in the TG/PL synthetic ratio (by 4%) after 3 days of cholesterol consumption may contribute to the mass ratio differences which are much greater. It also is possible that dietary cholesterol may affect the metabolic fate, eg. transport, of newly-formed TG. The 36-37% increase in the TG/PL biosynthetic ratio (Table 2) from 3 to 7 days can be compared with the mass ratio increase of 87-103% during the same period of time (5). The present results (Tables 1 and 2) suggest that gerbils consuming a diet containing 0.5% cholesterol may synthesize somewhat lesser amounts of PS relative to the other phospholipids. A slightly higher synthesis of PC relative to PE from 1,2-DG is suggested in livers of gerbils fed the 0.5% cholesterol diet for 3 but not for 7 days (Table 2). There are no analogous data in the literature with which to compare our results from [^{3}H]-glycerol as a function of dietary cholesterol supplementation.

The distribution of [^{3}H]-glycerol among the various molecular species of 1,2-DG, PC and PE in gerbil liver was generally similar to that reported in the rat, also using labelled glycerol (14,16). These data support the concept that the de novo pathways of PL synthesis produce predominantly oligoenoic (monoenoic plus dienoic) species of PC and PE plus hexaenoic species of PE (17). The much higher percentage of radioactivity found in the hexaenoic PE relative to the 1,2-DG, but not in the case of the corresponding PC, likely reflects, at least in part, a selectivity of the ethanolamine phosphotrans-

ferase but not the choline phosphotransferase for hexaenoic 1,2-DG as found in rat liver (11). The complement of molecular species of 1,2-DG, PC and PE formed de novo was not greatly influenced by dietary cholesterol. However, the mean relative rate of synthesis of tetraenoic to dienoic species of PC and PE was lower by 29 and 26%, respectively, in gerbils fed the cholesterol-supplemented diet; the mean percentage of incorporated radioactivity in the tetraenoic PC and PE was lower by 27 and 19%, respectively, in animals fed 0.5% cholesterol (Table 3). It appears that this altered de novo synthesis of particular species of PC and PE may account partly for the reduction in the 20:4/18:2 fatty acid ratio in these liver phospholipids when gerbils consumed a 0.5% cholesterol diet (5). It remains to be established if other metabolic pathways responsible for the entry of these polyunsaturated fatty acids into hepatic PC and PE, eg. transacylation reactions, may be influenced by dietary cholesterol.

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The Effect of Penicillin on Fatty Acid Synthesis and Excretion in *Streptococcus mutans* BHT

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ABSTRACT

Treatment of exponentially growing cultures of *Streptococcus mutans* BHT with growth-inhibitory concentrations (0.2 μg/ml) of benzylpenicillin stimulates the incorporation of [2-^{14}C] acetate into lipids excreted by the cells by as much as 69-fold, but does not change the amount of ^{14}C incorporated into intracellular lipids. At this concentration of penicillin cellular lysis does not occur. The radioactive label is incorporated exclusively into the fatty acid moieties of the glycerolipids. The increase in the radioactive content of the extracellular lipids reflects an actual net increase in the total fatty acid content as determined by a chemical assay. During a 4-hr incubation in the presence of penicillin, the extracellular fatty acid ester concentration (per mg cell dry weight) increases 1.5 fold, even though there is no growth or cellular lysis. No change is observed in the intracellular fatty acid ester content.

An indication of the relative rate of fatty acid synthesis was most readily obtained by placing *S. mutans* BHT in a buffer containing ^{14}C-acetate. Under these nongrowing conditions free fatty acids are the only lipids labeled, a factor which simplifies the assay. The addition of glycerol to the buffer causes all of the nonesterified fatty acids to be incorporated into glycerolipid. The cells excrete much of the lipid whether glycerol is present or not. Addition of penicillin to the nongrowth supporting buffer system does not stimulate the incorporation of [^{14}C]-acetate into fatty acids. However, if cells are exposed to penicillin in a growth-supporting medium and then are transferred to the nongrowing buffer system containing no penicillin, the previously exposed cells retain the ability to incorporate [^{14}C]-acetate into fatty acid at a higher rate than untreated cells over a prolonged period of time. The stimulation of [^{14}C]-acetate into fatty acids in this system parallels but is not dependent on the stimulation by penicillin of the incorporation of [^{14}C]-glycerol into glycerolipid and lipoteichoic acid synthesis previously demonstrated by our laboratory.

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INTRODUCTION

Despite the recent elucidation of the actual binding site of penicillin to the transpeptidase enzyme in *Streptomyces* R61 by X-ray crystallography by Kelly and coworkers (1), much of the mechanism by which penicillin brings about bacterial death remains unknown. One approach to understanding the mode of action of penicillin is to study the response of organisms that have become tolerant to penicillin. *Streptococcus mutans* BHT is a cariogenic bacterium which is said to be tolerant because it responds to penicillin in a bacteriostatic rather than bactericidal manner (2,3). We have shown previously that treatment of *S. mutans* BHT with penicillin results in a stimulation of de novo glycerolipid and lipoteichoic acid (LTA) synthesis and excretion in the absence of cellular lysis (4), even though exposure of *S. mutans* to penicillin results in a rapid inhibition of peptidoglycan, RNA, and protein synthesis in a concentration dependent fashion (5). Although several investigations (6,7,8) correlate antibiotic resistance with qualitative and quantitative differences in lipid composition of the bacterial envelope in different genera of gram-positive and gram-negative bacteria, the effect of these antibiotics on fatty acid synthesis has never been addressed. In this report we investigate whether penicillin promotes the net synthesis and excretion of fatty acids in *S. mutans* BHT.

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MATERIALS AND METHODS

S. mutans BHT was grown in a low sodium acetate (0.5 mg per ml), chemically defined medium (9) for at least eight generations in the presence of 2 μCi of [2-^{14}C]-acetate (New England Nuclear Corp., Boston, Massachusetts; specific activity 2.4 mCi/mmol) per ml and 0.01 M sodium bicarbonate in a total volume of 10 ml. A one ml aliquot of this culture was then inoculated into 30 ml of fresh chemically defined medium as described previously (9) containing [^{14}C]-acetate of the same specific activity. Culture turbidity was monitored at 675 nm in a Spectronic 21 colorimeter (Bausch and Lomb, Inc., Rochester, New York). Benzylpenicillin (GIBCO, Grand Island, New York, 1585 U/mg) at a concentration of 0.2 μg per ml (approximately 10 times the minimum inhibi-

tory concentration for this bacterium) was added to the exponentially growing culture at an A_{675} equivalent to 0.04. For experiments determining the effects of penicillin on the synthesis and excretion of [^{14}C] labeled fatty acids, duplicate 2-ml samples of the culture were collected at 60-min intervals via filtration on Whatman GF/C glass fiber filters (Whatman, Inc., Clifton, New Jersey) assuring complete separation of cells and media. The filters subsequently were washed twice with 5 ml of deionized, distilled water. Cells and media were extracted for lipid by the method of Bligh and Dyer (10). The chloroform soluble [^{14}C]-material was assayed for radioactivity in a liquid scintillation spectrometer with a 92% efficiency for ^{14}C (Tracor Analytic, Inc., Rockville, Maryland). These data are expressed as counts per min per μg dry weight of cells. The dry weight has been determined from the optical density which has been corrected for deviations from Beer's Law and is designated as the adjusted optical density (AOD) units (11) (1 AOD unit is equivalent to 0.39 μg of cellular dry weight per ml).

Deacylation of the [^{14}C-acetate]-labeled chloroform extracts was achieved by mild alkaline methanolysis as described by Ambron and Pieringer (12). Saponification of the chloroform soluble [^{14}C]-material also was performed according to the method of Volpe and Marasa (13) in the presence of one mg of stearic acid (Nutritional Biochemicals Corp, Cleveland, Ohio).

For the determination of the concentration of the [^{14}C]-acetate labeled fatty acids, an inoculum was prepared in the following fashion. A one-ml inoculum from a frozen stock containing a chemically defined medium (9) and 30% glycerol was transferred into 100 ml of low sodium acetate (0.5 mg per ml) defined medium (9) in the presence of 20 μCi of [2-^{14}C]-acetate (New England Nuclear; specific activity, 2.4 mCi/mmol) and 0.01 M sodium bicarbonate. This culture was allowed to grow to an A_{675} equivalent to 0.68. Then, 50-ml aliquots of this culture were inoculated into two separate flasks of fresh, low sodium acetate (0.5 mg per ml), chemically defined medium (500 ml) containing 0.2 μCi of [2-^{14}C]-acetate (New England Nuclear; specific activity, 2.4 mCi/mmol) per ml and 0.01 M sodium bicarbonate. At an A_{675} of 0.15, penicillin (0.2 μg per ml) was added to one of the 500-ml cultures. Coincident to the addition of penicillin, 250-ml aliquots of the cultures were harvested by centrifugation (1,860 x g, 20 min). The cells were washed twice with 50 ml of deionized, distilled water. Subsequently, the cells and media were extracted for lipid by the method of Bligh and Dyer (10). A second 250-ml sample of the culture was harvested by centrifugation (1,860 x g, 20 min) after a 4-hr incubation in the presence or absence of penicillin. Again, the cells were washed twice with 50 ml of deionized, distilled water. Lipid was then extracted from the cells and media (10). The [^{14}C]-labeled chloroform extracts were concentrated to dryness and resuspended in one ml of chloroform. The fatty acid ester determinations were performed in duplicate using a modification of the method of Stern and Shapiro (14). Monolaurin ester was used as a standard. Separate aliquots were taken and assayed for radioactivity in a liquid scintillation spectrometer.

The rate of incorporation of [^{14}C]-acetate into fatty acid was measured in a nongrowing cell system. Two separate cultures of exponentially growing *S. mutans* BHT in 30 ml of a chemically defined medium (9) containing 0.01 M sodium bicarbonate were permitted to grow to an A_{675} approximately equal to 0.28, at which time 0.2 μg per ml of penicillin was added to one of the cultures. After a 1.5-hr incubation in the presence or absence of the antibiotic, both cultures were harvested by centrifugation (1,860 x g, 20 min) and resuspended in 20 ml of buffer (pH 6.5) containing the following: sodium phosphate (monobasic, 2.05 mg per ml; dibasic 3.15 mg per ml), potassium phosphate (monobasic, 0.44 mg per ml; dibasic, 0.3 mg per ml), ammonium sulfate (120 mg per ml), and 1 μCi of [1-^{14}C]-acetate (New England Nuclear; specific activity, 2.4 mCi/mmol) per ml. In some experiments, the nongrowing cell system contained 20 μg per ml of glycerol in addition to the constituents listed above. At the designated intervals, duplicate 2-ml samples of cells were collected via filtration through Whatman GF/C glass fiber filters, again assuring complete separation of cells and media. After washing the filter twice with 5 ml of deionized, distilled water, the cells and media were extracted for lipid (10). An aliquot of the chloroform soluble fraction was assayed for radioactivity in a liquid scintillation spectrometer. Another portion of the [^{14}C]-acetate labeled lipid was chromatographed on thin layers (20 by 20 cm) of Silica Gel 250 (J.T. Baker Chemical Co., Phillipsburg, New Jersey) developed with either chloroform-methanol-water (65:25:4, v/v/v) or petroleum ether (high boiling)-ethyl ether-acetic acid (80:20:1, v/v/v) to the top of the plate. Authentic palmitic acid (Fischer Scientific Co., Fair Lawn, New Jersey) and (1[3] ^{14}C-glycerol) labeled lipids from *S. mutans* BHT or *S. faecalis* (faecium) ATCC 9790 prepared as previously described (4,15) were used

as standards. Intact lipids on TLC plates were detected by iodine vapor or, if radioactive, by scanning the plates in a recording gas flow Geiger counter.

RESULTS

Effect of Penicillin on the Incorporation of [2-^{14}C]-Acetate into Chloroform Soluble Material

Exposure of *S. mutans* BHT to 0.2 μg per ml of penicillin resulted in a rapid inhibition of growth approximately one hour after the addition of the antibiotic (4). The effect of penicillin on the incorporation of [^{14}C]-acetate into chloroform soluble material of *S. mutans* BHT is shown in Table 1. Although no significant differences were found in the amount of [^{14}C-acetate] labeled lipid per μg dry wt extracted from cells incubated in the presence or absence of penicillin, a greater than 50-fold increase of [^{14}C] labeled lipid was recovered from the medium after a 4-hr exposure period to the antibiotic. Thus, after a 4-hr incubation in the presence of penicillin, a 7.4-fold (317 cpm per μg dry wt for control in contrast to 2,358 cpm per μg dry wt for penicillin treated cultures) stimulation of [^{14}C]-acetate incorporation into total lipid (intra- and extracellular) per μg dry weight of cells was found. In contrast, the total amount of [^{14}C-acetate] labeled lipid (found intra- and extracellularly) from the corresponding untreated cultures remained relatively constant. These data emphasize the need to measure both extra- and intracellular lipid content when studying this response of tolerant bacteria to penicillin.

Effect of Penicillin on Fatty Acid Ester Concentration

Whether the stimulation of ^{14}C-acetate incorporation into lipid observed in Table 1 represents an actual net increase in lipid content during the suppression of growth by the penicillin was determined by measuring the fatty acid ester (14) concentration in *S. mutans* BHT at 0 and 4 hr of treatment with penicillin (0.2 μg/ml). The extracellular content of lipid fatty acid ester concentration increased more than 1.5-fold, but the intracellular lipid content did not change (Table 2). Thus there is a significant net increase in the amount of lipid made during this 4-hr period of treatment, and all of this newly synthesized lipid is excreted by the organism. However, the difference in the stimulation of incorporation of ^{14}C-acetate into lipid and the actual net increase in fatty acid content suggests that not all of the increase in incorporation of ^{14}C-acetate is due to net synthesis. It is clear that the tolerant cells do produce a net increase in lipid even though there is no growth, or RNA and DNA synthesis (5).

TABLE 1

Time Course of [2-^{14}C]-acetate Incorporation into the Lipids of *S. mutans* BHT Grown in the Presence and Absence of Benzylpenicillin[a]

	No penicillin					Penicillin (0.2 μg/ml)				
	Cells		Media			Cells		Media		
Hr	CPM/ml culture	CPM/μg cellular dry wt (A)	CPM/ml culture	CPM/μg cellular dry wt (B)	Sum (A+B)	CPM/ml culture	CPM/μg cellular dry wt (A)	CPM/ml culture	CPM/μg cellular dry wt (B)	Sum (A+B)
0	4,218 ± 98	313 ± 7.8	1,290 ± 127	103 ± 9.2	415 ± 1.4	5,087 ± 123	323 ± 7.8	1,331 ± 137	84 ± 8.5	407 ± 16.3
1	7,446 ± 492	234 ± 14.9	2,723 ± 321	85 ± 9.9	319 ± 5.0	8,814 ± 1,581	368 ± 57.0	9,582 ± 583	355 ± 21.0	723 ± 38.0
2	19,197 ± 3,133	295 ± 48.0	5,688 ± 1,242	88 ± 19.0	383 ± 67.0	8,264 ± 351	259 ± 11.0	28,944 ± 105	905 ± 3.5	1,163 ± 14.1
3	45,820 ± 959	323 ± 7.1	6,010 ± 504	42 ± 3.5	365 ± 11.0	8,367 ± 78.0	266 ± 8.5	47,594 ± 789	1,535 ± 25.5	1,801 ± 17.0
4	82,131 ± 3,345	286 ± 11.0	8,918 ± 139	30 ± 0.71	317 ± 11.0	8,670 ± 518	271 ± 15.6	66,789 ± 593	2,087 ± 18.4	2,358 ± 2.8

[a]These values represent the mean ± SD of two experiments performed in duplicate.
Cultures of *S. mutans* BHT were pre-equilibrated with [2-^{14}C]acetate before inoculation (1 ml) into 30 ml of fresh low sodium acetate (0.5 mg/ml) chemically defined medium containing 2 μCi of [2-^{14}C]acetate per ml. At an A_{675} equivalent to 0.04, benzylpenicillin at a concentration of 0.2 μg/ml was added to one of the exponentially growing cultures. Duplicate aliquots (2 ml) of the culture were collected via filtration at 60-min intervals, and the incorporation of [2-^{14}C]acetate into chloroform-soluble material was determined in cells and media.

TABLE 2

Change in Fatty Acid Ester Concentration Due to Penicillin

	μmol fatty acid[a]	mg dry wt of cells	total μmol fatty acid per mg dry wt
0 hr			
Intracellular	1.32 ± 0.38	16.63 ± 0.72	0.08 ± 0.02
Extracellular	5.89 ± 0.81		0.34 ± 0.04
		Total	0.42
4 hr			
Intracellular	1.51 ± 0.52	23.0 ± 1.39	0.07 ± 0.02
Extracellular	12.29 ± 2.52		0.53 ± 0.09
		Total	0.60

[a]The μmol fatty acid was determined from total chloroform extracts derived from 500-ml cultures which subsequently were evaporated to dryness and resuspended in 1 ml of chloroform.

These values represent the mean ± SD of two separate experiments each performed in duplicate.

Characterization of the [^{14}C-acetate] Labeled Chloroform Soluble Material

To determine if the [^{14}C]-acetate was incorporated only into the fatty acid moieties of lipids, the following experiments were performed. Deacylation under alkaline conditions of the lipids extracted into chloroform was performed as described by Ambron and Pieringer (12). After acidification of the deacylated [^{14}C] labeled chloroform extracts, equal volumes of chloroform and water were added, resulting in a two-phase system. Aliquots were taken from both the aqueous and chloroform phases and assayed for radioactivity. All of the radioactivity remained in the chloroform fraction. No radioactivity was detected in the aqueous phase. These data indicate that the [^{14}C]-acetate was incorporated solely into the fatty acid moieties of glycerolipids, and not into the deacylated derivatives of the phospho- and glycolipids.

Since another fate of the [^{14}C]-acetate label could be in the C_{55} isoprenoid compounds which are intermediates in bacterial cell wall biosynthesis, saponification of the [^{14}C] labeled chloroform soluble material was performed as previously described (13). After saponification and partitioning between petroleum ether and water the radioactivity that remained soluble in petroleum ether was the following: cellular lipids from control cells 252 cpm (out of a possible 56,000 cpm) and from penicillin-treated cells 324 cpm (out of a possible 94,000 cpm) and extracellular lipids from both control and penicillin-treated cells 0 cpm (out of a possible 31,000 and 878,000 cpm respectively). These data demonstrate that no appreciable amount of [^{14}C]-acetate was incorporated into the isoprenoid compounds of *S. mutans* BHT. Thus, the radioactive label from [^{14}C]-acetate is incorporated only into the fatty acid moieties of the lipids of *S. mutans* BHT.

Effect of Penicillin on the Rate of Incorporation of ^{14}C-acetate into Fatty Acid Measured in a Nongrowing Cell System

Several investigators (16-19) have established that antibiotics which inhibit the synthesis of cell wall induce the liberation of lipids into the culture medium. However, it has not been established whether these antibiotics cause the release of lipids due to a simple sloughing off of preformed fatty acids or if they stimulate the net synthesis and excretion of lipids. Our results, which show a net increase in lipid content, support the latter supposition. In an attempt to substantiate our initial finding, the following assay system was devised. Exponentially growing cultures of *S. mutans* BHT were harvested by centrifugation and subsequently resuspended in phosphate buffer containing [^{14}C]-acetate but lacking glucose. This system did not support growth but did allow the cells to incorporate [^{14}C]-acetate into either [^{14}C] labeled, nonesterified free fatty acids or by adding glycerol to the buffer [^{14}C] labeled fatty acids all esterified to the glycerol moiety of the phospho- and glycolipids. The [^{14}C]-acetate labeled glycerolipids synthesized in the nongrowth-supporting buffer containing glycerol comigrated with standard [^{14}C-glycerol] lipids prepared from *S. faecalis* (faecium) ATCC 9790 and *S. mutans* BHT *(S. mutans* and *S. faecalis* [faecium] have the same phospho- and glycolipids [6,15]) on thin layers of Silica Gel 250 developed with chloro-

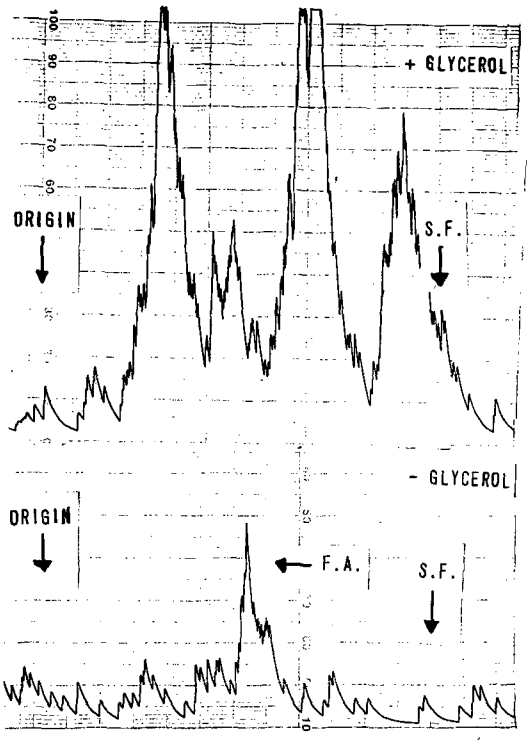

FIG. 1. Scans of the radioactive products synthesized in the nongrowing cell system in *S. mutans* BHT chromatographed on thin layers of Silica Gel 250 developed with chloroform-methanol-water (65:25:4, v/v/v).

form/methanol/water (65:25:4, v/v/v). The scan of these radioactive spots on the thin layer plate detected by a recording gas flow Geiger counter is shown in Figure 1. It is interestering to note that the same profile of glycerolipids (no free fatty acids present) is obtained when the cells are grown in a growth-supporting medium (for example, under the conditions described in Table 1). In contrast, in the absence of exogenous glycerol only one radioactive spot was detected on the thin layer plates when *S. mutans* BHT was incubated in the presence of only [^{14}C]-acetate, and this comigrated with authentic palmitic acid (Fig. 1). Thus, under these conditions of incubation the bacteria incorporate acetate into fatty acid but did not possess the ability to esterify the fatty acid to glycerophosphate unless exogenous glycerol was present. It should be noted that these cells were viable (tolerant to penicillin) and resumed growth when resuspended in a growth-supporting medium (data not shown).

Although caution should be exercised in equating incorporation of [^{14}C]-acetate into fatty acids with actual net synthesis of fatty acids in this nongrowing, whole cell system, the data suggest that the system may be of value in comparing the relative activity of fatty acid synthesizing enzymes in cells treated by various effectors. For example, the system should be useful in comparing the relative rate of incorporation of [^{14}C]-acetate into fatty acids in cells grown in the presence and absence of penicillin. Somewhat surprising and intriguing results were obtained (Table 3) when this experiment was carried out in the following way. Two exponentially growing cultures, one incubated in the presence of 0.2 μg per ml of penicillin for 1.5 hr, were harvested by centrifugation and washed. They were then resuspended in the nongrowing cell system containing only [^{14}C] acetate and lacking both penicillin and glycerol. Samples were taken and the cells and medium were extracted for lipid by the method of Bligh and Dyer (10). Subsequent resuspension of both of these cultures into growth media revealed that they were viable even after 3 hr in the absence of an energy source. Thus, exposure of cells to penicillin for 1.5 hr in a growth-supporting medium prior to their suspension in a ^{14}C-acetate-containing nongrowth-supporting medium resulted in a sustained rate of [^{14}C] acetate incorporation into fatty acids (Table 3) in cells that were no longer growing or exposed to penicillin. The rate of fatty acid synthesis in the untreated cultures was not sustained. This sustained rate of fatty acid synthesis in the penicillin-pretreated cultures ultimately results in an accumulation of free fatty acids excreted

TABLE 3

Pretreatment with Penicillin in Growth-Supporting Medium Prolongs High Rate of Incorporation of [^{14}C]-acetate into Fatty Acid in *S. mutans* BHT Transferred to a Buffer Containing No Penicillin

Time (min)	*No pretreatment* with penicillin (CPM/mg cellular dry wt/min)	*Pretreatment* with penicillin (CPM/mg cellular dry wt/min)
5	663 ± 362	1,065 ± 428
20	3,910 ± 794	4,929 ± 1,002
35	2,980 ± 1,313	5,437 ± 877
50	1,998 ± 867	5,355 ± 1,253
65	1,134 ± 685	4,931 ± 854

Cultures of *S. mutans* BHT growing exponentially in 30 ml of a chemically defined medium were treated with 0.2 μg/ml penicillin for 1.5 hr. The cells were harvested and resuspended in 20 ml of phosphate buffer containing 1 μCi/ml of [2-^{14}C]acetate. At the time intervals indicated, two-ml aliquots were taken and the lipids extracted. The total ^{14}C-lipid (intra- and extracellular) in the aliquots ranged from 1,200 to 70,000 CPM. The data are the mean ± SD of two experiments each carried out in duplicate. Controls were not exposed to penicillin.

TABLE 4

Lack of Stimulation of Fatty Acid Synthesis in *S. mutans* BHT by Penicillin in Non-growth Supporting Medium

	CPM/mg cellular dry wt/min			
	A		B	
Time (min)	- Penicillin	+ Penicillin	- Penicillin	+ Penicillin
5	1,592 (1,717) (1,467)	2,322 (2,178) (2,465)	561 (600) (522)	1,064 (1,148) (980)
65	1,963 (2,069) (1,857)	1,524 (1,619) (1,429)	643 (661) (624)	660 (690) (629)
125	1,387 (1,403) (1,371)	1,045 (1,096) (993)	364 (355) (373)	348 (329) (367)

Cultures of *S. mutans* BHT grown in a chemically defined medium (30 ml) were harvested by centrifugation and resuspended in buffer (20 ml) containing 1 μCi/ml of [2-^{14}C]acetate either lacking (-) or containing (+) 0.2 μg/ml of penicillin and without (A) or with (B) 20 μg/ml glycerol. At the designated times, duplicate 2 ml samples were taken and extracted for total lipid (CPM in aliquot ranged from 400 to 36,000). The experiment was carried out in duplicate.

into the culture medium. The [^{14}C] labeled fatty acids synthesized in this system comigrated with authentic palmitic acid on thin layers of Silica Gel 250 developed with petroleum ether/ethyl ether/acetic acid (80:20:1, v/v/v). This system does not separate individual fatty acids from each other.

In order to rule out the possibility that the prolonged synthesis of fatty acids was due to the presence of a residual amount of penicillin not completely washed out from the cells, the following experiment was performed. Two exponentially growing cultures of *S. mutans* BHT were harvested by centrifugation and resuspended into the nongrowing cell system, one containing 0.2 μg per ml of penicillin in addition to [^{14}C]-acetate (one contained glycerol). Pencillin had no effect on fatty acid synthesis or glycerolipid synthesis in the nongrowing cell system, as seen in Table 4. These data demonstrate that the stimulation and prolongation of the incorporation of [^{14}C]-acetate into fatty acids by penicillin requires the presence of a growth-supporting, energy-providing culture system.

DISCUSSION

The release of lipid from bacteria into the culture medium following exposure to cell wall inhibiting antibiotics has been well established (16-19). In 1973, Nakao and coworkers (18) and Kikuchi et al. (16) reported the extracellular accumulation of phospholipid by penicillin treated *Corynebacterium alkanolyticum*. Subsequently, other investigators (17,19-21) also demonstrated the excretion of lipid into the growth medium upon exposure to cell wall inhibiting antibiotics in several bacterial species including *Bifidobacterium bifidum* subsp. *pennsylvanicus, Streptoccus pneumonia, Staphylococcus pyogenes,* and *Streptococcus sanguis.* Moreover, increased incorporation of radioactive lipid precursors has been shown after penicillin treatment in streptomycin-resistant *S. aureus* H (22), autolysin-defective mutants of *S. faecium* (23), and *B. bifidum* (17). However, the relationship between the net synthesis of fatty acids and the excretion of lipid from the cell had not been fully appreciated. One of the more obvious features of the data of this paper is that the stimulation of the net incorporation of [^{14}C]-acetate into fatty acids by penicillin requires that the *S. mutans* BHT be supplied with a growth-supporting medium. An external energy source is not needed for fatty acid synthesis itself, because once stimulated the cells continue to produce fatty acids at relatively high rates for more than an hour in a nongrowth-supporting medium (phosphate buffer containing a very small amount of ^{14}C-acetate). The most likely source of energy for fatty acid synthesis in the latter system is the intracellular polysaccharides stored in relatively large amounts by *S. mutans* BHT (24). Since penicillin does not stimulate fatty acid synthesis in the absence of a growth-supporting medium, it seems likely that some intermediary factor is involved either directly or indirectly (e.g. required for generation of intracellular polysaccharides) in the mechanism of action of penicillin.

Penicillin causes a net increase (1.5-fold) in the total amount of lipid generated by the organism. Since there is little growth in the cells, perhaps it is not surprising to find that almost all of this lipid is excreted into the medium. This

excretion is not related to cell lysis because *S. mutans* BHT do not lyse under these conditions of treatment with penicillin (24).

It is interesting to note that perturbations in lipid biosynthesis caused by penicillin occur in gram-positive organisms that, although susceptible to growth inhibition, are tolerant to the lytic or lethal effects of penicillin. For example, a greater content of diphosphatidylglycerol has been reported in *Bifidobacterium bifidum* subsp. *pennsylvanicus* (19), penicillin-resistant *S. aureus* (6), *S. sanguis* (21) and *S. mutans* BHT (4) (both of these organisms are tolerant to the lytic consequence of penicillin), and autolysis-defective mutants of *S. faecium* (23) following treatment with penicillin. The stimulation of lipoteichoic acid and lipid synthesis and the qualitative alterations in lipid composition in *S. mutans* BHT after penicillin treatment (4) correlated well with other reports on the lipid content of antibiotic tolerant organisms (6-8). This finding may prove to be significant in view of the demonstration that in addition to inhibition of peptidoglycan synthesis, RNA and protein synthesis are inhibited as well in *S. mutans* strains after treatment with penicillin (5). Stimulation of lipid synthesis and excretion was concurrent with inhibition of peptidoglycan, and protein synthesis after treatment with cell wall inhibiting antiobitics in *B. bifidum* (17). Recently, Rogers and coworkers (25) also reported the inhibition of protein and peptidoglycan synthesis with a transient stimulation of lipid synthesis following β-lactam addition in an autolysin-deficient *B. subtilis*. However, the β-lactams were shown to be bactericidal in these organisms. It is also interesting to note that the β-lactams were determined to be hydrophilic based on their partition coefficients in n-octanol and 0.05 M sodium phosphate (26). This then suggests that a coordinated stimulation of hydrophobic lipid and amphipathic lipoteichoic acid synthesis and excretion may play a role in the tolerant response of some gram-positive bacteria to the lytic or lethal consequences of the β-lactam antibiotics.

ACKNOWLEDGMENTS

This manuscript is dedicated to Eugene P. Kennedy on the occasion of his 65th birthday. Discussions with Dr. G.D. Shockman are gratefully acknowledged. This work was supported by Public Health Service research grant AI-05730.

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Red Pigment-Forming Substances from Autoxidized Linolenate: Identification of Prostaglandin-like Substances[1]

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ABSTRACT

The chemical structures of lipid degradation products capable of reacting with amino acids and forming red pigments were investigated. The red pigment-forming substances (RPS's) derived from autoxidized linolenate in triglyceride of linseed oil were purified successively by gel chromatography on Sephadex LH-20, column chromatography and TLC on Silica gel 60, and HPLC on μ-Porasil. Consequently, three types of RPS's were isolated.

IR spectra of RPS's were similar, except for slight differences in the fingerprint region (1300-650 cm^{-1}). These substances included the OH group (3500 cm^{-1} region), conjugated aldehyde (νC=O 1688 cm^{-1}, νC=C 1635 cm^{-1}) and ketone (νC=O 1740 cm^{-1}) in their molecules. RPS's were analyzed by GLC and GC-MS after derivatization with dimethylhydrazine and/or trimethylsilyl reagents, before and after the reduction with $NaBH_4$ and/or hydrogenation with PtO_2. The fragmentation patterns indicated the presence of an ethyl group in addition to the functional groups described above, and the molecular formula was estimated to be $C_{10}H_{14}O_3$. Further elucidation of the structures was obtained by ^{13}C- and ^1H-NMR analyses, and evidence was obtained for the presence of a hydroxypentanone ring, a PG-like structure. The sequence of the protons on the ring carbons was unequivocally deduced from the double resonance experiments.

All the data taken together suggested that the RPS's were the stereoisomer of 3-(2-ethyl-5-hydroxy-3-oxo) cyclopentanyl-2-propenal.

Lipids 20:180-186, 1985.

INTRODUCTION

Interactions between peroxidized lipids and nitrogenous compounds result in undesirable changes: off-flavor (1-3), diminished nutrient content (4-6), polymerization (7-10), accelerated formation of brown pigments in foods (11,12), loss of enzyme activities (13-15) and disorder or disease states in biological systems (16,17).

Conjugated carbonyls and hydroxyl or hydroperoxyl conjugated carbonyls, which were highly reactive to amino acid, were identified from autoxidized methyl linoleate (18), methyl linolenate (19) and linseed oil (20) as a part of the study on discoloration of fatty foods. In the course of these studies, we observed the production of lipid degradation products which could form reddish pigments by reaction with amino acids. Further study (21) of these compounds showed the discoloration property and the universal occurrence of the red pigment-forming substances (RPS's) produced by the autoxidation of polyenoic fatty acids with more than three double bonds. A purified RPS from autoxidized methyl linolenate had an absorption maximum at 226-227 nm in ethanol, and reddish pigments produced by the reaction with amino acids had an absorption maximum at 510-520 nm. RPS's, which are highly reactive to amino compounds as well as the conjugated carbonyls described above, may play a role in discoloration of fatty foods and in lipid peroxidation in vivo.

I now report the chemical structures of RPS's derived from autoxidation of linolenate in triglyceride of linseed oil.

[1]Presented at the General Meeting of JSSF held in Tokyo University of Fisheries, Tokyo, April 1983.

MATERIALS AND METHODS

Preparation of RPS's

Autoxidation and Extraction. The triglyceride (TG) purified from commercial linseed oil (Nakarai Chem. Co.) by column chromatography on silicic acid (22) was oxidized, in the dark, with stirring and occasional bubbling with air until half of the octadecatrienoic acid in the TG was oxidized (7 days at 40 C). The fatty acid composition of the TG was as follows: C18:3, 62%; C18:2, 18%; C18:1, 13%; C16:0, 5%, and C18:0, 2%. The autoxidized TG (100g) was extracted three times with 250 ml portions of 90% MeOH, and the solvent of the combined extract was removed under reduced pressure.

Gel Chromatography. The extract was fractionated by gel chromatography on Sephadex LH-20 (Pharmacia Fine Chem. Co.) with $CHCl_3$: MeOH (1/1, v/v) and the eluted position of RPS's was monitored according to the discoloration with glycine, as reported previously (21). The RPS-fraction was pooled for further purification.

Silicic acid chromatography. The RPS-frac-

tion from the gel chromatography was separated on a Silicagel 60 prepacked column (24 x 1 cm, E. Merck Co.) with $CHCl_3$:MeOH (97/3, v/v) as the eluent. RPS's eluted were monitored again, according to the discoloration with glycine.

High performance liquid chromatography (HPLC). The crude RPS-fraction obtained by silicic acid chromatography was further separated by HPLC on a μ-Porasil column (30 x 0.78 cm, Waters Assoc. Inc.) eluted with n-hexane:2-propanol (9/1, v/v) at 4.0 ml/min, and with four subfractions, R'-O~R'-3, were recovered. RPS-fractions (R'-1, R'-2, R'-3) were re-chromatographed on the same column, separately, until each of the isolated fractions gave a single peak. Consequently, three R-1, R-2 and R-3 were isolated (yield 0.005%≧).

Thin layer chromatography (TLC). Preparative and analytical TLC were carried out on Silica gel 60 plates with a fluorescent indicator (E. Merck Co.) in benzene:acetone:EtOH (70/30/2, v/v/v). The spots on the TLC plate were visualized under UV light (254 nm), and RPS-spots were detected by spraying 0.5 M glycine (pH 7.0) and subsequent heating at 45 C in an electric oven for 20~30 min.

Characterization of RPS

Preparation of Derivatives. Dimethyl hydrazone (DMH) and trimethylsilyl (TMS) derivatives of RPS's were prepared by the methods described previously (20,23,24). The RPS's were reduced with $NaBH_4$ and hydrogenated with PtO_2, as a catalyst (20).

GLC Analysis. DMH and/or TMS derivatives of RPS's were analyzed using a Shimadzu GC-4BPF gas chromatograph equipped with glass columns (2 m x 4 mm) and packed with 5% SE-30. Flow rate of the N_2 carrier gas was 40 ml/min, and the temperature was linearly programmed from 130 to 220 C at 3 C/min.

IR Analysis. IR spectra were measured in chloroform with an EPI-G21 Hitachi IR spectrometer.

GC-MS Analysis. Low resolution mass spectra were obtained with a combined GC-MS spectrometer (Hitachi RM-50 GC) equipped with a 5% SE-30 column. Ionizing voltage was 20 eV. A Hitachi M-80 GC-MS spectrometer was used for the high resolution mass spectrometry.

NMR Analysis. ^1H- and ^{13}C-NMR spectra were obtained on a JEOL JNM-FX100 Fourier transform spectrometer, in deuterated chloroform. Chemical shifts are given as δ value in ppm downfield from the internal standard, tetramethylsilane signal.

RESULTS

The crude RPS-fraction obtained by silicic acid chromatography was further purified by HPLC (Fig. 1-I), and RPS's in pooled subfractions (R'-O~R'-3) were monitored on a TLC plate. As shown in Figure 1-II, three different RPS's which changed from colorless to pink by spraying the glycine solution were evident, namely R-1 (upper spot, Rf 0.33), R-2 (lower spot, Rf 0.28) and R-3 (lower spot, Rf 0.28). R-2 and R-3 have the same Rf in Fig. 1-II, but

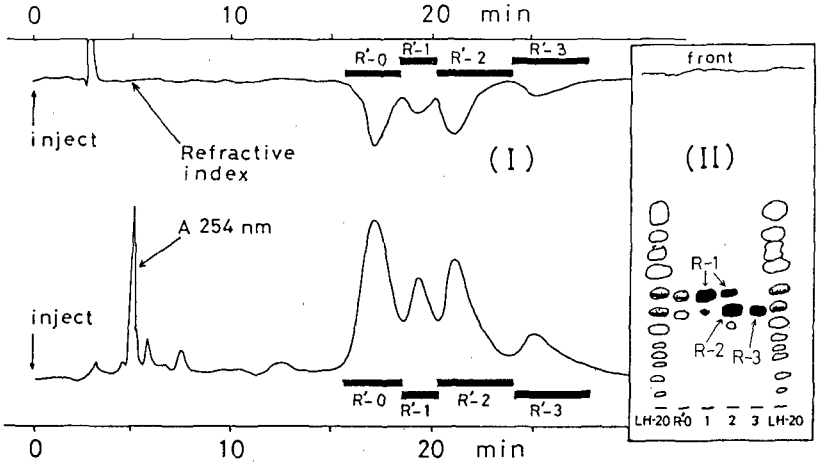

FIG. 1. (I) High performance liquid chromatography of the RPS-fraction prepared by silicic acid chromatography. See text for details. (II) Thin layer chromatography of HPLC-subfractions. All the spots were detected under UV-light (254 nm). The dark spots indicate RPS's with a pink color after spraying with the glycine solution.

they are different compounds as they had different eluting positions on the HPLC. Furthermore, R-2 was less stable and a part of R-2 became R-1 when the R′-2 fraction was subjected to preparative TLC on Silica gel 60 F_{254}. On the other hand, since R-1 and R-3 were relatively stable, they could be purified by preparative TLC following HPLC on the μ-Porasil column.

IR spectra of the purified RPS's measured in chloroform are shown in Figure 2. These are similar, except for slight differences in the fingerprint region (1300-650 cm^{-1}). The absorption bands at 3500 cm^{-1} region indicated the presence of an OH group (not OOH group because of a peroxide negative reaction on the TLC plate [25]). The two strong absorption bands at 1740 cm^{-1} and 1688 cm^{-1} were due to C=O stretching, and could be assigned to ketone and conjugated aldehyde, respectively (26,27). The small absorption band at 1635 cm^{-1} was due to C=C stretching of the double bond of conjugated aldehyde (27). Thus, it can be estimated that the RPS's possessed one OH and two kinds of carbonyl groups.

After successive derivatization with DMH- and TMS-reagents, the derivatives of RPS's were analyzed by GLC. As shown in the case of R-1 (Fig. 3-I), two prominent peaks (A and B) were observed. The amount of peak-A decreased with corresponding increases in the amounts of peak-B, when the time of derivatization with the DMH-reagent was extended. Mass spectra of peaks-A and -B are shown in Figure 4 (I and II). Although the molecular ion of peak-A was M^+296 and that of peak-B was M^+338, the fragmentation patterns closely resembled each other. Namely, the base peaks are 123, and prominent ions at M-15 (-CH_3), M-44 (-$N[CH_3]_2$), M-90 (-HOTMS). M-44-90, m/e 152 and m/e 59 are observed in both spectra. The molecular weights measured by high resolution mass spectrometry were A:296.1909 and B:338.2509, and the molecular formulas were estimated to be peak-A: $C_{15}H_{28}O_2N_2Si_1$ and peak-B:$C_{17}H_{34}O_1N_4Si_1$, respectively. These results indicate that peak-A had one DMH and one TMS group and one underivatized carbonyl group. Peak-B had two DMH and one TMS groups in the molecule. In other words, the mother compounds of peaks-A and -B were the same, and peak-A was estimated to be an incomplete, free ketone derivative.

After the reduction with $NaBH_4$ and subsequent derivatization with TMS-reagents, the TMS's of RPS's (R-1, R-2, R-3) were analyzed by GLC and GC-MS. As shown in Figure 3-II, only peak-a was detected when R-1 was the main component, and the same held for R-2. On the other hand, almost the same amounts in splitting peaks (a and b) were detected in R-3

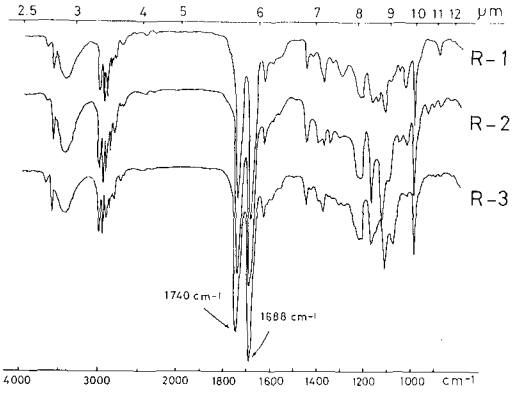

FIG. 2. Infrared spectra of RPS's. Spectra were measured in chloroform solution (0.1 mm thickness cell). R-1, R-2 and R-3 were main components of R′-1, R′-2 and R′-3, respectively, as shown in Figure 1-II.

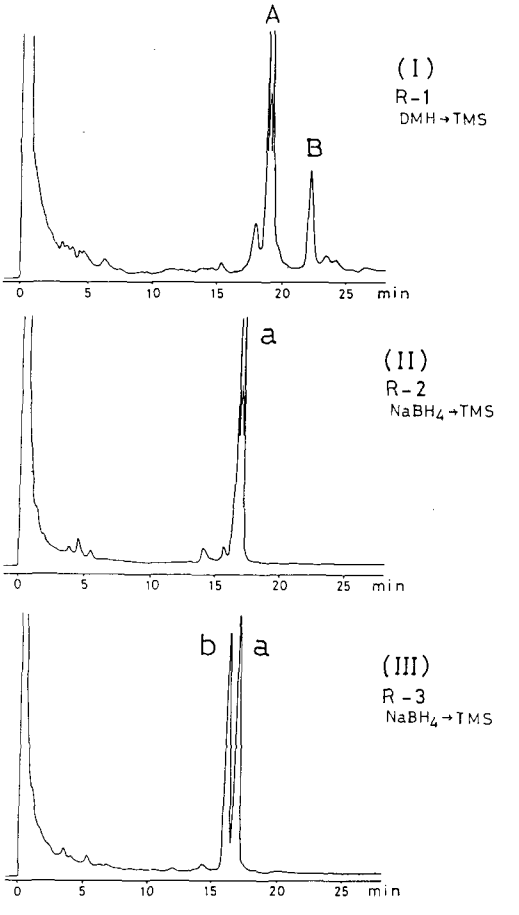

FIG. 3. Gas chromatography of dimethylhydrazone (DMH) and/or trimethylsilyl (TMS) derivatives of RPS's. (I), DMH-TMS derivatives of R-1; (II); TMS derivative of R-2 after the reduction with $NaBH_4$; (III), TMS derivatives of R-3 after the reduction with $NaBH_4$.

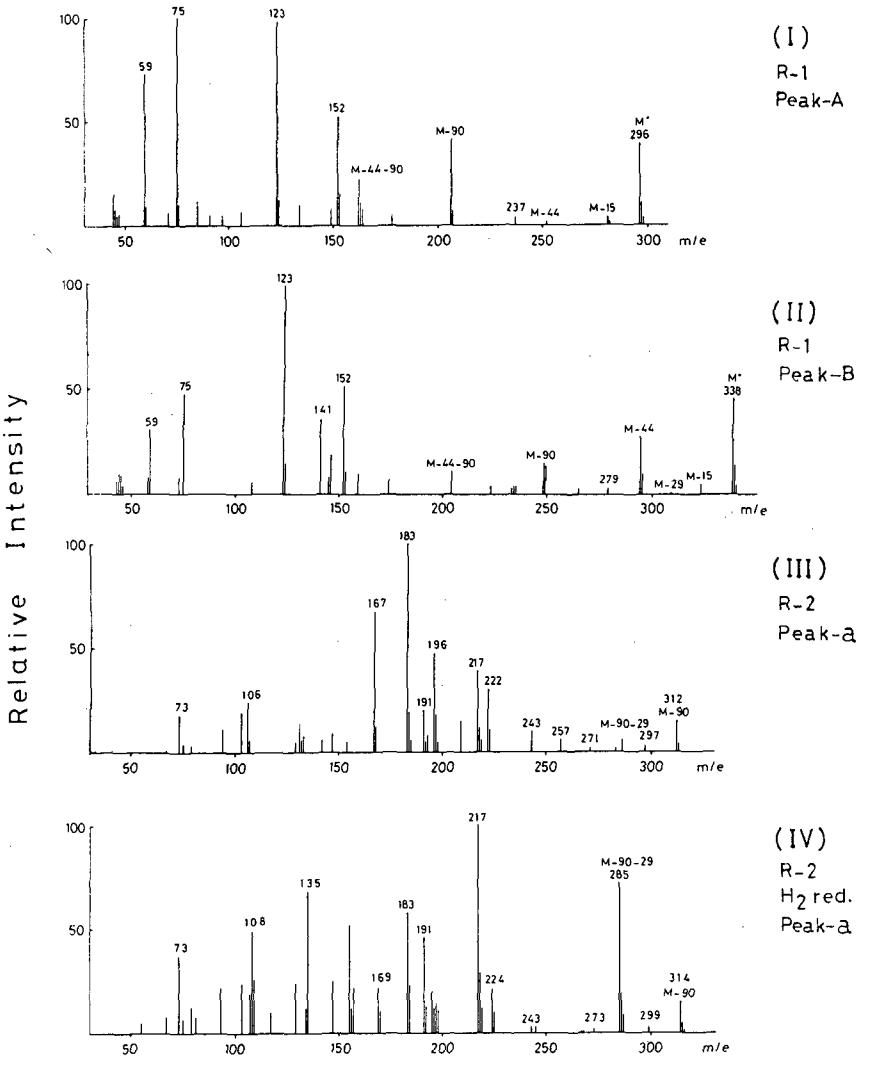

FIG. 4. Mass spectra of derivatized RPS's. (I), peak-A in Figure 3-I; (II),peak-B in Figure 3-I. (III), peak-a in Figure 3-II; (IV), TMS derivative of derived R-2 obtained by the reduction of $NaBH_4$ and subsequent hydrogenation.

(Fig. 3-III). The mass spectra of peaks-a and -b could not be differentiated. The fragmentation pattern of peak-a is shown in Figure 4-III. The base peak is m/e 183, and the largest ion detected is m/e 312 (M-HOTMS). Having been reduced with $NaBH_4$, R-2 was hydrogenated and derivatized with TMS-reagents. The mass spectrum of the derivative is shown in Figure 4-IV. The largest ion of m/e 312 in Figure 4-III is increased to m/e 314, and several similarly increased ions (m+2/e) were detected, although some series of ions remained unchanged. These results indicate that only one double bond is present in the carbon chain in the molecule, and that this double bond probably is conjugated with the aldehyde group. M-29 in Figure 4-II and M-90-29 in Figures 4-III and IV indicate the presence of a branched ethyl group in the molecules. Judging from the results described above, it was estimated that the RPS's (R-1, R-2, R-3) were stereoisomers with the molecular formula of $C_{10}H_{14}O_3$; they had one conjugated aldehyde, one ketone, one OH and one side chain of the ethyl group, and a ring structure in the molecule is deduced.

Further evidence for the presence of the ring structure in RPS molecules was obtained using ^{13}C- and ^{1}H-NMR analyses. The carbon chemical shifts of R-1, R-2 and R-3 are given in Table 1. The carbon chemical shifts of C-1,

TABLE 1

^{13}C-Chemical Shifts of RPS's

Carbon number[a]	Proton noise decoupling[b] R-1	R-2	R-3	Off-resonance decoupling[c]
C-1	193.4	193.2	193.1	d (=CH-)
C-2	134.6	136.6	134.7	d (=CH-)
C-3	155.5	152.4	155.8	d (=CH-)
C-4	50.5	55.4	55.6	d (=CH-)
C-5	69.8	69.9	71.6	d (=CH-)
C-6	48.2	45.0	46.6	t (-CH_2-)
C-7	216.4	214.4	—[d]	s (=C=)
C-8	50.2	49.2	53.7	d (=CH-)
C-9	20.7	19.3	20.7	t (-CH_2-)
C-10	10.9	12.2	11.0	q (-CH_3)

[a]Carbon numbers of proposed structure in Figure 5.

[b]Chemical shifts in ppm downfield from tetramethylsilane.

[c]Signals under proton decoupling condition were split into q: quadruplet; t: triplet; d: doublet; s: singlet.

[d]Due to an inadequate amount of the sample, a clear signal was not observed.

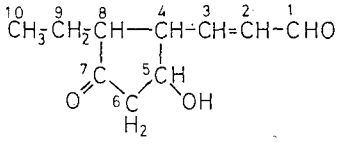

FIG. 5. Proposed structure of RPS's.

C-2, C-3 and C-9, C-10 indicated the presence of conjugated aldehyde (2-propenal) and ethyl groups, respectively, in each molecule. The results of off-resonance decoupling suggested the presence of a hydroxypentanone ring, and the chemical shifts of the ring carbons are in close agreement with those of prostaglandin E and D groups (28-30). As a result, these spectral data, including IR and MS data, support the tentative structure shown in Figure 5. Further evidence for the validity of the chemical structure proposed was obtained by ^{1}H-NMR analysis (Fig. 6). The sequence of protons on the ring carbons was unequivocally deduced from double resonance experiments on R-1. Namely, by starting with irradiation at 51.1842 KHz (1.63 ppm, CH_3-$\underset{9}{CH_2}$-) or at 51.8773 KHz (9.56 ppm, -$\underset{1}{CH}$O), each series of adjacent protons of the carbon chain was determined successively; with irradiation at 51.3013 KHz (2.84 ppm, -$\underset{4}{CH}$-), the splittings of proton resonances on adjacent carbons (-$\underset{8}{CH}$-, -$\underset{5}{CH}$O-, =$\underset{3}{CH}$-) collapsed. In a similar manner, adjacent protons of -$\underset{8}{CH}$- were $CH_3C\underset{9}{H_2}$- and -$\underset{4}{CH}$-, and of -$\underset{5}{CH}$O- were -$\underset{4}{CH}$- and -$\underset{6}{CH_2}$-. Thus, the validity of the proposed structure in Figure 5 was confirmed. Namely, RPS's were determined to be the stereoisomer of 3-(2-ethyl-5-hydroxy-3-oxo) cyclopentanyl-2-propenal.

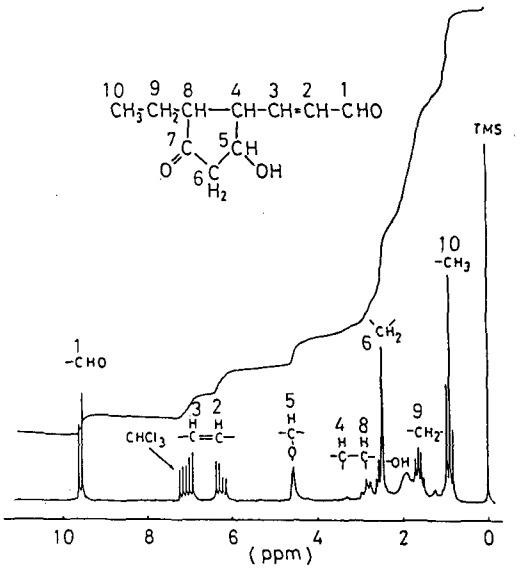

FIG. 6. ^{1}H-NMR spectra of RPS (R-I).

DISCUSSION

A proposed scheme for the formation of RPS's from linolenate is illustrated in Figure 7. Abstraction of a hydrogen atom from the double allylic methylene groups of C-11 or C-14 produces pentadienyl radicals. Oxidation at either end of these pentadienyl radicals forms 9-, 12-, 13- and 16-monohydroperoxy radicals. The hydroperoxy radicals which have a double bond at β-γ carbons (13- and 12-isomers) can only be cyclized to form PGG-like endoperoxides. Since the work of D.H. Nugteren, H. Vonkeman and D.A. Van Dorp (31), the formation of the bicyclo endoperoxides during autoxidation of polyenoic fatty acid was recognized by several groups of workers (32-37). However, little is known of the destiny of these unstable compounds during autoxidation, except for the data on the malondialdehyde or TBA-reactive substance (32,38). With subsequent scissions of the endoperoxide and in the position α to the carbon bearing peroxyl group, RPS's of PGD- or PGE-like structure can be produced; that is, two types of RPS's (I and II) can be

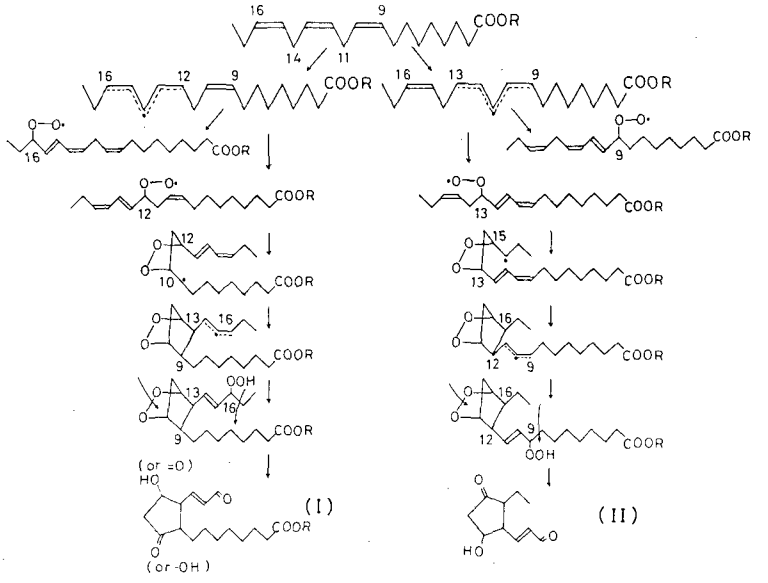

FIG. 7. A proposed scheme for the formation of RPS's.

formed from 12- and 13-isomers, respectively. As described in the results, only type II was identified in this experiment because the TG of linseed oil was used as the starting material. Production of type-II and other related compounds from methyl linolenate will be described in a following paper. Since RPS's of type II have three assymetric centers and a double bond, production of 16 different stereoisomers can be expected, although there may be stereochemical preferences. In the present experiment, three different stereoisomers (R-1, R-2, R-3) were detected. The stereoconfiguration of these isomers is the subject of ongoing studies.

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Dr. M. Toyomizu, Emeritus Professor of Kyushu University gave advice and encouragement; Dr. E. Kuwano assisted with the NMR spectra, K. Seike gave technical assistance and M. Ohara commented on the manuscript.

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METHODS

High Performance Liquid Chromatographic Separation of Stereoisomeric Bile Acids as their UV-Sensitive Esters

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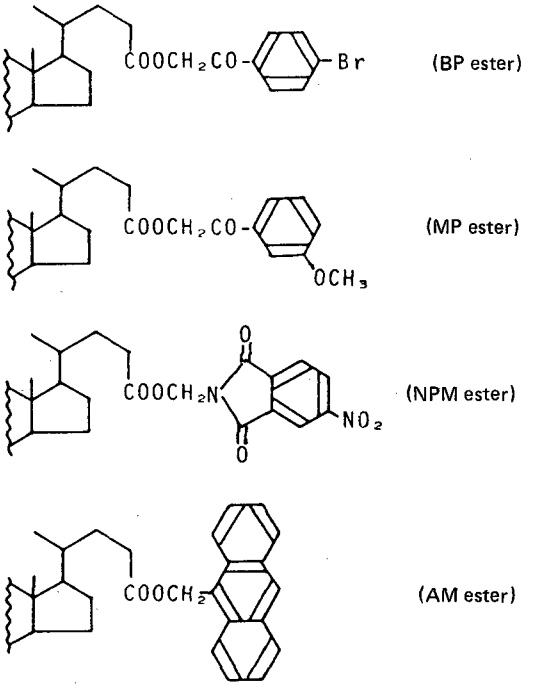
ABSTRACT

High performance liquid chromatographic separation of a series of mono-, di- and trihydroxylated 5β-cholanic acids, which differ only in position and configuration of hydroxyl groups at positions C-3, C-7 and/or C-12, is reported. The C-24 free acids were derivatized to four different classes of UV-sensitive esters, i.e., *p*-bromophenacyl (BP), *m*-methoxyphenacyl (MP), 4-nitrophthalimidemethyl (NPM) and 9-anthrylmethyl (AM) esters, and chromatographed on two variants of C_{18} reversed-phase columns (Nova-Pak C_{18} and Zorbax ODS) with methanol-water systems as mobile phase. Separation efficiency and elution order of some isomeric pairs were influenced by both the structure of the C-24 ester groups and the nature of the columns used. Excellent chromatographic properties were found for those derivatives, particularly for the NPM esters.
Lipids 20:187-194, 1985.

INTRODUCTION

High performance liquid chromatography (HPLC) has been used for the separation and quantitation of bile acids (5β-cholanic), as well as of their glycine and taurine conjugates and sulfates present in physiological fluids (1-12). Although the free acids have been analyzed directly in HPLC with detectors based on refractive index or ultraviolet absorption at low wave length (below 210 nm), these detectors are inadequate for micro analysis: refractive index detectors because of their intrinsically low sensitivity, and the UV detectors because carboxylic acids possess weak chromophores with absorption maxima at wave lengths where the detector is least reliable. Therefore, reagents have been introduced successfully introduced which form UV-sensitive derivatives of carboxylic acids with both higher sensitivity and improved chromatographic properties (13-18). Among these derivatives are the esters, *p*-bromophenacyl (4,5,15), *m*-methoxyphenacyl (MP) (16), 4-nitrophthalimidemethyl (NPM) (17), and 9-anthrylmethyl (AM) (18). Several of these esters of common bile acids have been shown to be markedly superior to the use of free acids or their alkyl esters in HPLC analysis (4,5,14,15,18).

The availability of the complete set of the 26 theoretically possible stereoisomers of 5β-cholanic acid having one to three hydroxyl groups at positions 3, 7 and 12 has prompted us to compare the four types of esters of these acids for analytical efficiency in HPLC. Accordingly, we present in this paper the preparation of the BP, MP, NMP and AM esters (Scheme 1) of the 26 acids, plus those of the 3α,6α and



SCHEME 1. Type of the C-24 UV-sensitive esters derivatives prepared.

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3α,6α,7α acids, and a comparison of their chromatographic properties on reversed-phase columns.

EXPERIMENTAL

Samples and Reagents

All samples used in this work were from collections in our laboratories (19-21).

The labeling reagents, i.e., *p*-bromophenacyl bromide, *m*-methoxyphenacyl bromide, N-chloromethyl-4-nitrophthalimide and 9-chloromethylanthracene, were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan) and used without further purification. 18-Crown-6 and 10% tetramethylammonium hydroxide solution as catalysts were obtained from Aldrich (Milwaukee, Wisconsin, U.S.A.) and Wako Pure Chemical Industries Ltd. (Osaka, Japan), respectively. All the other chemicals were of analytical grade. Thin layer chromatography (TLC) plates pre-coated with silica gel $60F_{254}$ (20 x 20 cm, 0.25 mm layer thickness) were obtained from Merck (Darmstadt, W. Germany). Solvents used were of HPLC grade and were degassed by sonication prior to use.

Instruments

M.p. were determined on an electrical micro hot stage and are uncorrected. ^{1}H-NMR spectra were obtained on a JEOL FX-90Q instrument, with $CDCl_3$ containing 1% Me_4Si as the solvent, except where otherwise indicated. Chemical shifts are expressed in δ ppm relative to Me_4Si.

HPLC apparatus used was a Waters M-45 solvent delivery system (Waters Assoc., Milford, Massachusetts, U.S.A.) equipped with a Model U6K sample loop injector and a Model SPD-2A UV detector (Shimadzu Corp., Kyoto, Japan); the wavelength selected for all measurements was 254 nm. Nova-Pak C_{18} (15 cm x 3.9 mm I.D., 5 μm; Waters Assoc.) and Zorbax ODS (25 cm x 4.6 mm I.D., 5-6 μm; Du Pont Co., Wilmington, Delaware, U.S.A.) reversed-phase columns were used under ambient conditions. Methanol/water mixtures (ratio from 95:5 to 70:30, v/v) were used as mobile phases at flow rates of 0.5 to 1.5 ml/min.

Derivatization Procedure

(Method A). To a solution of bile acid (ca. 0.4 mg; 1 μmole) in 200 μl of methanol in a tapered reaction vial was added 3 μl of 5% methanolic potassium hydroxide. After standing at room temperature for 5 min, the solvent was evaporated under N_2. To the residual potassium salt dissolved in 200 μl of acetonitrile was added ca. 0.5 mg (2 μmoles) of labeling reagent (*p*-bromophenacyl bromide, *m*-methoxyphenacyl bromide or N-chloromethyl-4-nitrophthalimide) and ca. 0.25 mg (1 μmole) of 18-crown-6. The vial was capped, heated and stirred at 60 C for 20 min. After cooling, the solution was filtered (0.45 μm: Millipore Corp., Bedford, Massachusetts, U.S.A.), washed with 500 μl of methanol, and an aliquot (1-2 μl) of the filtrate was injected directly into the HPLC.

(Method B). To a solution of bile acid (ca. 0.4 mg; 1 μmole) in 100 μl of 10% tetramethylammonium hydroxide (10 μl) in DMF (1 ml) was added 200 μl of 9-chloromethylanthracene (12 mg; 50 μmole) in cyclohexane (5 ml) in a reaction vial. The mixture was capped and stirred for 30 min at 70 C. After cooling, the solution was treated as described in method A.

(Method C). To a solution of 160 mg (0.4 mmoles) of lithocholic acid in 4 ml of methanol was added 0.4 ml of 5% methanolic potassium hydroxide. The mixture was stirred for 5 min, most of the solvent was evaporated, and the residual potassium salt was redissolved in 4 ml of acetonitrile. To this solution was then added 250 mg (1 mmole) of labeling reagent (*p*-bromophenacyl bromide, *m*-methoxyphenacyl bromide or N-chloromethyl-4-nitrophthalimide) and 14 mg (50 μmoles) of 18-crown-6, and the mixture was stirred for 1 hr at 70 C. The reaction product was extracted with CH_2Cl_2 (X2), and the combined extract was washed with water, dried with Drierite, and evaporated to a residual oil which, when treated with a suitable solvent, crystallized.

(Method D). To a solution of 160 mg (0.4 mmoles) of lithocholic acid dissolved in 5 ml of DMF was added a solution of 2 ml of 10% tetramethylammonium hydroxide in 5 ml of cyclohexane and a solution of 185 mg (0.8 mmoles) of 9-chloromethylanthracene in 7 ml of cyclohexane. The mixture was stirred for 1 hr at 80 C and the reaction product was processed as described in method C.

RESULTS AND DISCUSSION

Preparation of the C-24 UV-Sensitive Esters of the Bile Acids

Four different classes of the C-24 esters of bile acids were prepared in the manners described in the Experimental section. Method A was used for preparing the BP, MP and NPM esters by the use of 18-crown-6 ether (22) as catalyst and *p*-bromophenacyl bromide, *m*-methoxyphenacyl bromide, and N-chloromethyl-4-nitrophthalimide, respectively, as labeling reagents. In these derivatizations crown ether was found to be a better catalyst than either triethylamine (4) or N,N-diisopropylethylamine (16); yields were excellent under milder

TABLE 1

Physical Data for the C-24 Ester Derivatives of Lithocholic Acid

Ester	m.p. [C] (solvent)	R_f-value[a]	^{1}-H-NMR (ppm)[b]
BP	99-101 (MeOH)	0.40	0.66 (3H,s,C-18Me), 0.92 (3H,s,c-19Me), 3.60 (1H,brm,C-3CHOH), 5.27 (2H,s,COOCH$_2$CO-), 7.70 (4H,q,j=9Hz,phenyl protons)
MP	175-176 (acetone-hexane)	0.31	0.64 (3H,s,C-18Me), 0.91 (3H,s,C-19Me), 3.57 (1H,brm,C-3CHOH), 3.85 (3H,s,OCH$_3$), 5.32 (2H,s,COOCH$_2$CO-), 7.13-7.46 (4H,m,phenyl protons)
NPM	164-167 (acetone-hexane)	0.42	0.64 (3H,s,C-18Me), 0.90 (3H,s,C-19Me), 3.53 (1H,brm,C-3CHOH), 3.62 (2H,s,COOCH$_2$-)
AM	78-80 (aq. MeOH)	0.41	0.64 (3H,s,C-18Me), 0.91 (3H,s,C-19Me), 3.60 (1H,brm,c-3CHOH), 3.66 (2H,s,COOCH$_2$-), 7.31 (9H,s,phenyl protons)

[a]Developed in hexane/EtOAc (60:40; v/v)

[b]MP and NPM esters were measured in $CDCl_3$ containing 20% DMSO-d_6. s, singlet; brm, broad multiplet; m, multiplet; q, quartet.

conditions. However, with 9-chloromethylanthracene as labeling reagent, the esterification by the use of crown ether as catalyst was incomplete. The AM esterification was completed by using tetramethylammonium hydroxide as catalyst (method B) (18). Both methods A and B are suitable for complete esterification of small amounts (< 1 mg) of the acids. The labeled esters are stable for at least several months under refrigeration. The purity of each ester derivative was checked by TLC (23) before use.

Methods C and D, suitable for preparing larger amounts (> 100 mg) of the esters, were used to prepare the four types of esters of lithocholic (3α-OH) acid for characterization (Table 1).

Resolution of the UV-Sensitive Esters by HPLC on C-18 Columns

Figure 1 is a chromatogram of a mixture of the four different esters of lithocholic acid on a Nova-Pak C_{18} column, with methanol/water (90:10, v/v) as eluent. The esters were eluted in the order of NPM, MP, BP and AM, with the AM ester having much longer retention time.

Figure 2 shows the clean separation of a mixture of the NPM esters of five common bile

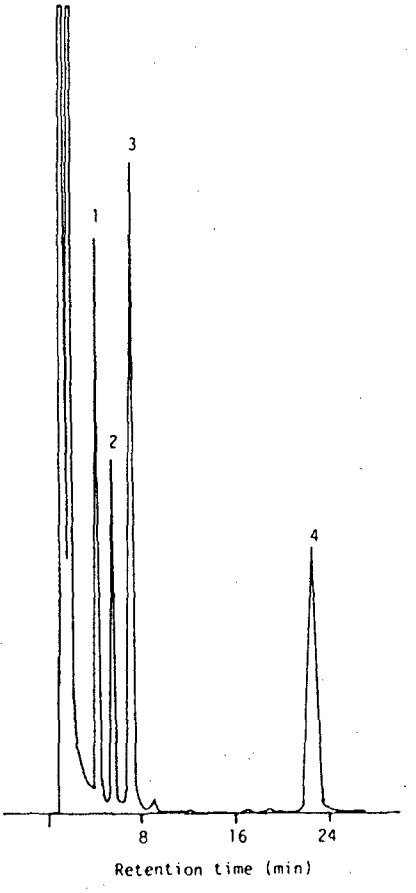

FIG. 1. HPLC of a mixture of the C-24 esters of lithocholic acid. Conditions: column, Nova-Pak C_{18} (5 μm); mobile phase, methanol/water (90:10); flow rate, 1.0 ml/min; detector, UV at 254 nm. Peak identification; 1 = NPM, 2 = MP, 3 = BP, 4 = AM.

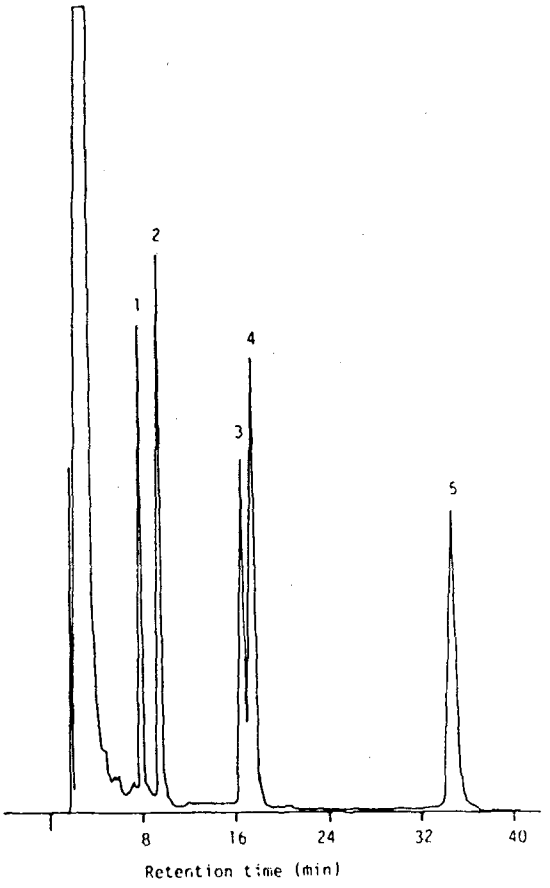

FIG. 2. HPLC of a mixture of five common bile acids as their NPM esters. Conditions: column, Zorbax ODS (5-6 μm); mobile phase, methanol/water (85:15); flow rate, 1.0 ml/min; detector, UV at 254 nm. Peak identification, position and configuration of hydroxyls: 1 = 3α,7β, 2 = 3α,7α,12α, 3 = 3α,7α, 4 = 3α,12α, 5 = 3α.

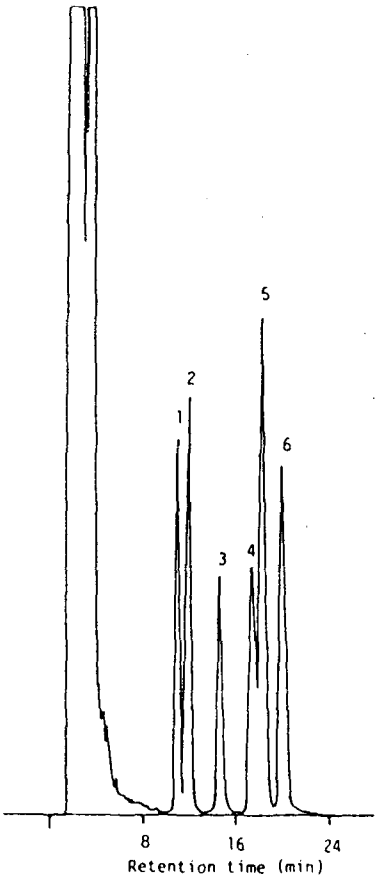

FIG. 3. HPLC of a mixture of six monohydroxylated bile acid isomers as their MP esters. Conditions: column, Nova-Pak C_{18} (5 μm); mobile phase, methanol/water (90:10); flow rate, 0.5 ml/min; detector, UV at 254 nm. Peak identification, position and configuration of hydroxyls: 1 = 3β, 2 = 3α, 3 = 7β, 4 = 7α, 5 = 12α, 6 = 12β.

TABLE 2

Relative Capacity Factor (rk′) of the C-24 Ester Derivatives of Monohydroxylated 5β-Cholanic Acids on Two Reversed-Phase Column[a]

	Nova-Pak C_{18}				Zorbax ODS			
Compounds	BP (90:10) (0.5)	MP (90:10) (0.5)	NPM (90:10) (0.5)	AM (95:5) (1.0)	BP (90:10) (1.0)	MP (90:10) (1.0)	NPM (90:10) (1.0)	AM (95:5) (1.5)
3β-OH	0.90	0.90	0.88	1.09	0.95	0.94	1.00	1.23
3α-OH	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
7β-OH	1.23	1.27	1.39	1.20	1.14	1.14	1.34	1.14
7α-OH	1.51	1.55	1.61	1.31	1.39	1.38	1.54	1.27
12α-OH	1.61	1.64	1.61	1.31	1.48	1.44	1.54	1.23
12β-OH	1.80	1.82	2.03	1.60	1.66	1.60	1.94	1.50

[a]Ratios of the capacity factors of lithocholic acid (3α) esters to those of bile acid esters: values in parentheses refer to volumetric composition of methanol/water and flow rate (ml/min).

TABLE 3

Relative Capacity Factor (rk′) of the C-24 Ester Derivatives of Dihydroxylated 5β-Cholanic Acids on Two Reversed-Phase Columns[a]

Compounds	Nova-Pak C_{18} BP (80:20) (1:0)	MP (75:25) (1.0)	NPM (75:25) (1.0)	AM (85:15) (1.0)	Zorbax ODS BP (80:20) (1.5)	MP (80:20) (1.0)	NPM (80:20) (1.0)	AM (90:10) (1.0)
3α,7β-$(OH)_2$	0.29	0.27	0.33	0.36	0.30	0.32	0.36	0.45
3β,7β-$(OH)_2$	0.31	0.29	0.35	0.45	0.34	0.36	0.39	0.56
3β,7α-$(OH)_2$	0.49	0.48	0.50	0.62	0.54	0.55	0.54	0.72
3α,7α-$(OH)_2$	0.89	0.89	0.92	0.96	0.90	0.91	0.94	1.00
3α,12β-$(OH)_2$	0.44	0.39	0.50	0.56	0.46	0.45	0.53	0.62
3β,12β-$(OH)_2$	0.44	0.39	0.53	0.60	0.44	0.45	0.56	0.68
3β,12α-$(OH)_2$	0.49	0.48	0.50	0.60	0.56	0.55	0.53	0.64
3α,12α-$(OH)_2$	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
7β,12β-$(OH)_2$	0.21	0.19	0.28	0.25	0.24	0.19	0.32	0.35
7β,12α-$(OH)_2$	0.27	0.25	0.33	0.28	0.29	0.24	0.36	0.36
7α,12β-$(OH)_2$	0.33	0.29	0.38	0.41	0.36	0.36	0.41	0.49
7α,12α-$(OH)_2$	0.49	1.12	1.19	1.00	0.56	1.12	1.20	1.00
3α,6α-$(OH)_2$	0.41	0.39	0.43	0.51	0.44	0.45	0.47	0.61

[a]Ratios of the capacity factors of deoxycholic acid (3α,12α) esters to those of bile acids: see footnote to Table 2 for other explanations.

adids, i.e., lithocolic, deoxycholic (3α,12α), chenodeoxycholic (3α,7α), ursodeoxycholic (3α,7β) and cholic (3α,7α,12α) acids, on a Zorbax ODS column with methanol/water (85:15, v/v) as eluent. The corresponding BP and MP esters were similarly well resolved. As compared with published HPLC work in which free acids (3,8) or their methyl esters (1) were analyzed, the superiority in the use of the UV-sensitive esters is well demonstrated. (When the solvent mixture is low in water percentage [ca. 10%], excess labeling reagent and reaction by-product usually emerge early and do not interfere with bile acid peaks of interest, so that a preliminary purification step is avoided. However, with esters of the more polar dihydroxy and trihydroxy acids, which require higher percentages of water in the mobile phase [ca. 20-30%] for good resolution, contaminating signals can interfere with early-emerging ester peaks. Among the four types compared in this study, the NPM esters were the best in this respect [Fig. 4]. For circumventing the problem of interfering early contaminants a gradient elution technique would be useful [4]).

Tables 2-4, respectively, show the retention data for the C-24 ester derivatives of 6 monohydroxy, 13 dihydroxy and 9 trihydroxy 5β-cholanic acids on two variants of C_{18} reversed-phase columns, together with eluent system and flow rates. The retention data were expressed as the relative capacity factors (rk′), since the values are more reproducible between laboratories and less dependent on operating parameters (9) (see below concerning limitations). Inspection of the tables revealed that the capacity factors of each compound respond dramatically to minor structural differences in the 5β-steroid nucleus, and that separation efficiency and elution order of some isomeric pairs are influenced by both the structure of the C-24 ester groups and the nature of columns used.

As shown in Figure 3, the six monohydroxylated bile acids differing in position and stereochemistry are well resolved as their BP and MP ester derivatives on both Nova-Pak C_{18} and Zorbax ODS columns, emerging in the order of 3β-, 3α-, 7β-, 7α-, 12α- and 12β-ols. This order is inconsistent with the previous generalization (24) that the 3α-equatorial hydroxyl group in 5β-steroids is more polar than the 3β-axial. A similar anomaly was reported recently in the HPLC analysis of the C-3 benzoate derivatives of epimeric 5α-steroids (25). However, the AM ester of the 3α-hydroxy compound is eluted before its 3β-epimer in accord with the above generalization.

When a single mobile phase was used, separation of the 13 stereoisomeric dihydroxy esters was incomplete (Fig. 4), this being true for all four ester types, regardless of whether the column was Nova-Pak or Zorbax. However, the stereoisomers of each group of 3,7 or 7,12 esters were well separated on both columns, while the four in the 3,12 group as their BP and AM esters could be only partially resolved, with the Zorbax

TABLE 4

Relative Capacity Factor (rk') of the C-24 Ester Derivatives of Trihydroxylated 5β-Cholanic Acids on Two Reversed-Phase Columns[a,b]

Compounds	Nova-Pak C_{18}				Zorbax ODS			
	BP (75:25) (1.0)	MP (70:30) (1.0)	NPM (70:30) (1.0)	AM (75:25) (1.0)	BP (80:20) (1.0)	MP (75:25) (1.0)	NPM (75:25) (1.0)	AM (85:15) (1.5)
3α,7β,12β-$(OH)_3$	0.06	0.04	0.04	0.12	0.10	0.07	0.09	0.11
3β,7β,12β-$(OH)_3$	0.08	0.05	0.06	0.14	0.12	0.09	0.12	0.13
3β,7β,12α-$(OH)_3$	0.12	0.10	0.09	0.18	0.16	0.14	0.15	0.15
3β,7α,12β-$(OH)_3$	0.14	0.11	0.12	0.25	0.19	0.16	0.19	0.20
3α,7β,12α-$(OH)_3$	0.20	0.17	0.18	0.25	0.25	0.22	0.24	0.20
3α,7α,12β-$(OH)_3$	0.24	0.19	0.24	0.38	0.30	0.33	0.30	0.28
3β,7α,12α-$(OH)_3$	0.47	0.44	0.40	0.53	0.51	0.49	0.47	0.38
3α,7α,12α-$(OH)_3$	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
3α,6α,7α-$(OH)_3$	0.93	0.96	1.00	1.07	1.00	1.00	1.04	0.77

[a]Ratios of the capacity factors of cholic acid (3α,7α,12α) esters to those of bile acid esters: see footnote to Table 2 for other explanation.

[b]The rk' values of BP esters determined (Chang, F.C., Iida, T., and Brannan, S., unpublished) on an Excalibar ODS column, methanol/water (80:20, v/v; flow rate, 1.5 ml/min) = 3α,7β,12β, 0.23; 3β,7β,12β, 0.25; 3β,7β,12α, 0.29; 3β,7α,12β, 0.32; 3α,7β,12α, 0.37; 3α,7α,12β, 0.42; 3β,7α,12α, 0.60; 3α,7α,12α, 1.00.

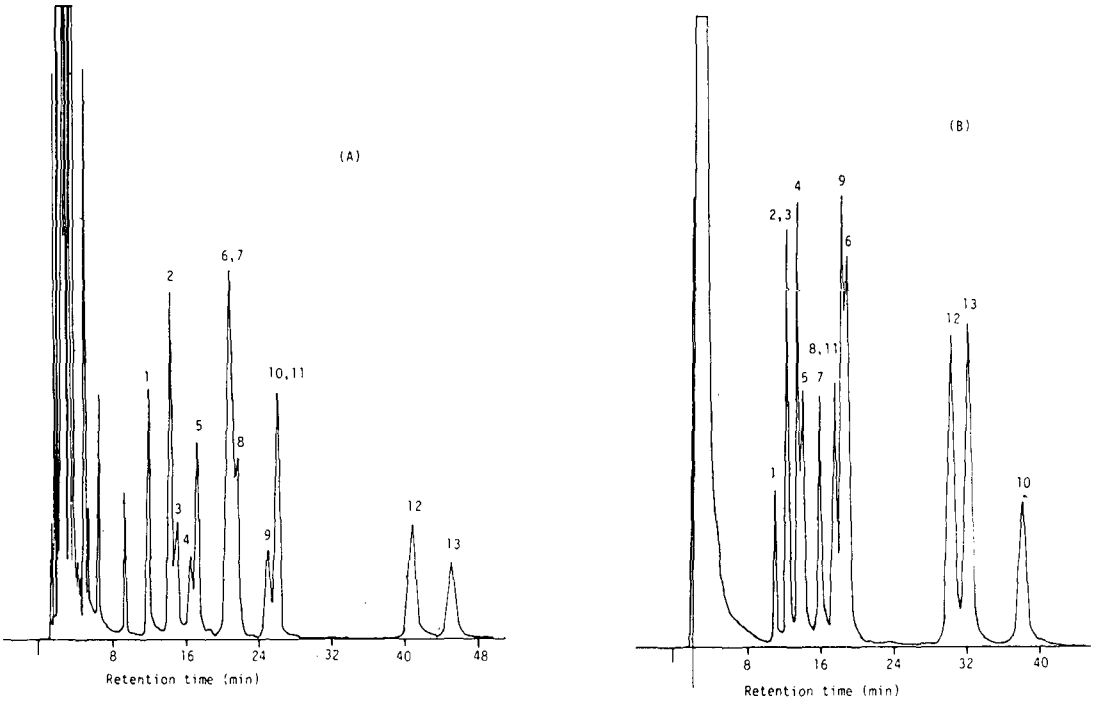

FIG. 4. HPLC of a mixture of 13 dihydroxylated bile acid isomers as their (A) BP and (B) NPM esters. Conditions: column, Zorbax ODS (5-6 μm); mobile phase, methanol/water (80:20); flow rate, 1.5 ml/min for A and 1.0 ml/min for B; detector, UV at 254 nm. Peak identification, position and configuration of hydroxyls: 1 = 7β,12β, 2 = 7β,12α, 3 = 3α,7β, 4 = 3β,7β, 5 = 7α,12β, 6 = 3β,12β, 7 = 3α,6α, 8 = 3α,12β, 9 = 3β,7α, 10 = 7α,12α, 11 = 3β,12α, 12 = 3α,7α, 13 = 3α,12α.

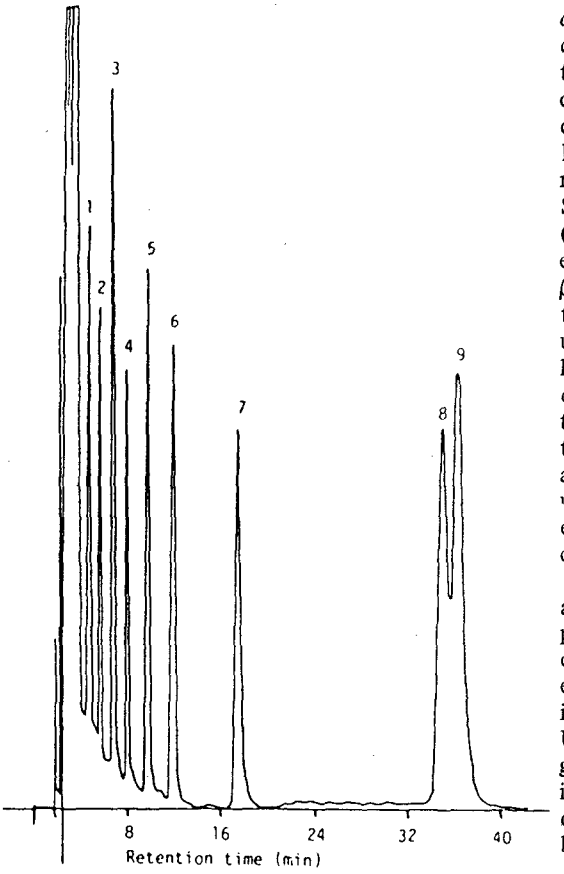

FIG. 5. HPLC of a mixture of nine trihydroxylated bile acid isomers as their NMP esters. *Conditions*: column, Zorbax ODS (5-6 μm); mobile phase, methanol/water (75:25); flow rate, 1.0 ml/min; detector, UV at 254 nm. Peak identification, position and configuration of hydroxyls: 1 = 3α,7β,12β, 2 = 3β,7β,12β, 3 = 3β,7β,12α, 4 = 3β,7α,12β, 5 = 3α,7β,12α, 6 = 3α,7α,12β, 7 = 3β,7α,12α, 8 = 3α,7α,12α, 9 = 3α,6α,7α.

column giving slightly better resolution. (In previously unpublished work [Chang, F.C., Iida, T., and Brannan, S.] done on an Excalibur ODS 3 μ column [methanol/water, 80:20, v/v; flow rate, 0.5 ml/min], the 12 BP esters with the exception of the 3β,7β and 7α,12β pair were well resolved, and by a change of solvent to acetonitrile/methanol/water [40:40:20, v/v/v; flow rate, 1.5 ml/min], the recalcitrant pair was nicely separated; 3β,7β before 7α,12β.)

The eight stereoisomeric 3,7,12-acids are completely separated as their BP, MP and NPM esters, emerging from each column in the order, $\alpha\beta\beta$, $\beta\beta\beta$, $\beta\beta\alpha$, $\beta\alpha\beta$, $\alpha\beta\alpha$, $\alpha\alpha\beta$, $\beta\alpha\alpha$, $\alpha\alpha\alpha$, precisely corresponding to the order found with their methyl esters (Reference 20, footnote 14). As seen in Table 4, although the order of elution of the eight esters of each type from the two columns is the same, the relative capacity factors (rk' values) are dissimilar. Additional evidence that rk' values are not reproducible on different columns, as suggested by Shaw, Rivetna and Elliott (9), is found in a previous resolution (Chang, F.C., Iida, T., and Brannan, S., unpublished data) of the eight BP esters. (See Table 4, footnote b.) Of the four types of esters compared, the AM esters of the $\alpha\beta\alpha$ and $\beta\alpha\beta$ acids were unresolved on both columns, although the elution order of the compounds was unchanged. The four corresponding esters of hyocholic (3α,6α,7α) acid were included in this comparison for added interest. Table 4 shows that both the rk' values and order of elution of the two sets of esters of cholic and hyocholic acids vary according to the columns used. Figure 5 is a typical chromatogram of the NPM esters of the nine trihydroxy acids on a C-18 column.

A high degree of HPLC resolution was achieved for a number of difficult-to-separate pairs of bile acid isomers by derivatizing their carboxy groups to form BP, MP, NPM or AM esters. These esters, because of the simplicity in their preparation and the enhancement of UV-sensitivity, should be useful in the investigation of unknown bile acid metabolites present in biological extracts. Of the four types of derivatives examined, the NPM esters appear to be the most promising.

ACKNOWLEDGMENT

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Tocopherol-Phospholipid Liposomes: Maximum Content and Stability to Serum Proteins

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ABSTRACT

This study addresses two questions: 1) what is the maximum amount of tocopherol that can be contained in egg phosphatidylcholine liposomes, and 2) what is the stability of these vesicles in the presence of serum proteins? These liposomes, made with a French pressure cell, can contain no more than 33 mol % of tocopherol. Tocopherol changes liposomes in a manner similar to cholesterol, making them larger, less permeable to aqueous dyes and highly resistant to protein-induced disruption. The suppression of protein-induced disruption is more pronounced with tocopherol than with cholesterol, even at lower molar ratios. Thus, liposomes containing alpha tocopherol (15 to 30 mol %) may be useful for delivering physiological quantities of this vitamin to cells in culture or to tissues in vivo. *Lipids* 20:195-200, 1985.

INTRODUCTION

Tocopherol is an antioxidant vitamin. Deficiency of this substance is associated with a variety of species-specific disorders, including degeneration of myelinated axons in both the central and peripheral nervous systems of mammals (1,2,3). Tocopherol-containing liposomes may prove useful for the treatment of neurological symptoms associated with certain disease states (abetalipoproteinemia, biliary atresia, cystic fibrosis) in which tocopherol depletion is prominent (4,5,6). Previous work (7) has shown that tocopherol-enriched human lipoproteins enhance the survival of developing rat neurons in vitro. The role that tocopherol plays in this phenomenon may be explored by using tocopherol-containing liposomes to introduce the water-insoluble vitamin to aqueous media.

The purpose of this communication is to define a simple and reproducible method for incorporating large amounts of the vitamin into egg phosphatidylcholine (PC) liposomes using a French pressure cell and to test the feasibility of these liposomes as transport vesicles in vivo by assessing their interactions with serum proteins. Others have produced tocopherol-phospholipid liposomes by sonication (8,9,10). However, the reported yield of incorporated tocopherol was much lower than found in our study.

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MATERIALS AND METHODS

Egg phosphatidylcholine (egg PC) (Sigma, St. Louis, Missouri) was purified as previously described (11). Cholesterol (Nutritional Biochemicals, Cleveland, Ohio) was recrystallized three times from methanol. Both *d*-alpha tocopherol (αT) and 4(5)-carboxyfluorescein (CF) were used as received (Eastman Kodak, Rochester, New York). Fetal calf serum (FCS) (Sterile Systems, Logan, Utah) was obtained through the cell culture facility at the University of California, San Francisco. Rat plasma apo high density lipoprotein (apo HDL) was prepared as described (12).

Protein was determined by the method of Lowry et al. (13), with bovine serum albumin as standard. Cholesterol was determined by an enzymatic method (14). Lipid phosphorus was measured according to Bartlett (15). Alpha tocopherol was measured by fluorescence (16).

Liposomes were prepared from an aqueous dispersion of lipids as described previously (11). Briefly, 20 mg of egg PC in ethanol were mixed with ethanolic αT to form solutions containing between 5 and 67 mol % of tocopherol. Aqueous dispersions, made by adding 3 ml of column buffer (0.2 M NaCl, 0.02% Na azide, 0.001 M EDTA, pH 7.6) to the dried lipids, were passed rapidly three times through a French pressure cell (3/8" piston diameter; American Instrument Co., Silver Springs, Maryland) at 20,000 psi. Gel chromatography was performed on certain preparations using a 2% agarose (BioRad, Richmond, California) column (95 x 1.2 cm).

The flasks used for evaporation were later rinsed in buffer and dried. Three ml of ethanol were then added to each flask in order to quantitatively recover any lipids that had not entered the aqueous phase.

To make CF-containing liposomes, 2 ml of an aqueous (0.2 M) solution of CF was used in place of column buffer. Either tocopherol or cholesterol in ethanol was mixed with ethanolic egg PC and dried as usual. The aqueous dispersions were run four times through the French pressure cell at 20,000 psi. The resulting liposomes were filtered through a Sephadex G-50 column (2 × 10 cm) to remove external dye (12). A single cholesterol value of 37 mol % was chosen because previous studies (12) had shown that this concentration produced the most stable liposomes with respect to apolipoprotein-induced disruption.

The fluorescence of CF is largely self-quenched at concentrations above 0.10 M whereas in dilute solutions fluorescence is proportional to the dye concentration (17). Therefore, an increase in fluorescence of dilute suspensions of liposomes was interpreted as evidence of either increased membrane permeability (slow dye release, measured in minutes, in the absence of protein) or liposomal disruption (rapid dye release, measured in seconds, in the presence of protein or detergents) (12).

Fluorescence was measured on a spectrophotofluorometer (Model SPF-500, American Instrument Co.) at 37 C, 460 nm excitation and 520 nm emission (2 nm slit widths). One ml of either standard buffer or buffer plus serum proteins was put into quartz cuvets and warmed to 37 C. Ten μl of CF-containing liposomes were then added and rapidly mixed (5-6 seconds elapsed time). The intensity of fluorescence was continuously monitored. At the end of the experiment, 0.1 ml of a 20% solution of Triton X 100 was added to disrupt the liposomes and release the dye remaining within them. The percent of dye released at time t, R(t), was calculated according to the following formula:

$$R(t) = 100\,(F(t)-F_o)/[(F_{Max} \times 1.1)-F_o]. \quad [1]$$

Where F_o is the intensity of fluorescence of 1 ml of buffer plus 10 μl of dye-containing liposomes at time 0; F(t) is the fluorescent intensity of 1 ml of buffer or serum proteins plus 10 μl of dye-containing liposomes at time t; and F_{Max} is the fluorescent intensity of the above mixtures after the addition of 0.1 ml of Triton solution. The relative values for F_{Max} on the day of preparation were 90, 98, 80 and 66 fluorescent units for the four different types of liposomes made (pure PC; 37 mol % cholesterol; 15 mol % tocopherol and 29 mol % tocopherol, respectively). After six weeks of storage at 4 C, the liposomes were warmed to 37 C and examined on the same spectrophotofluorometer using identical settings. The resulting F_{Max} values were 118, 120, 86 and 66 fluorescent units respectively.

TABLE 1

Percentage of Trapped Dye Released After Storage at 4 C

Lipsome type	Background fluorescence	
	Day of preparation	After 6 weeks
100 mol % egg PC	9.4%	43%
PC + 37 mol % cholesterol	7.2%	28%
PC + 15 mol % α-tocopherol	1.2%	2.3%
PC + 29 mol % α-tocopherol	1.5%	3.1%

The background fluorescence (F_B) of CF from several different liposome preparations (warmed to 37 C) is calculated according to Equation [2]. The F_B found on the day of preparation is compared to the F_B found following 6 weeks of storage at 4 C under an argon atmosphere.

The background fluorescence, F_B, at time t = 0 was calculated by the following equation:

$$F_B = 100\,[F_o/(F_{Max} \times 1.1)] \quad [2]$$

Between 1 and 10% of the total maximum fluorescence was found in F_B (Table 1) on the day of preparation.

Negatively stained preparations of liposomes were prepared and sized as described previously (11). They were examined and photographed at 60,000 magnification and 80 KV in a Siemens 101 electron microscope (Siemens Corp. Medical/Industrial Groups, Iseline, New Jersey).

RESULTS

Maximum Molar Ratio of αT to PC in Aqueous Dispersions

Ethanolic solutions of αT and PC containing from 5 to 67 mol % tocopherol were dried and dispersed in 3 ml of column buffer. The amount of each lipid entering the aqueous phase was then compared to the quantity adhering to the walls of the evaporating flask. When the starting ratio of αT was less than or equal to 20 mol %, nearly all the lipid went into the aqueous dispersion (Fig. 1). However, as the initial αT concentrations were increased, the entry of both αT and PC into the aqueous phase was reduced because the lipids began to adhere to the glass walls of the evaporating flask. At an initial

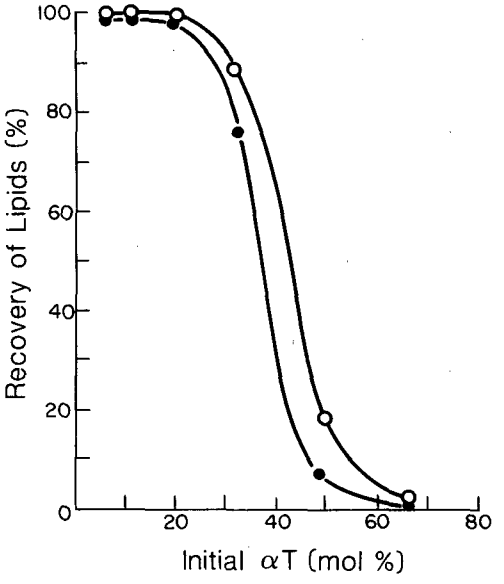

FIG. 1. The percent recovery of both αT (•) and PC (○) in the aqueous dispersion is shown as a function of the starting ratio of the lipids in ethanolic solution. These data were averaged from duplicate assays.

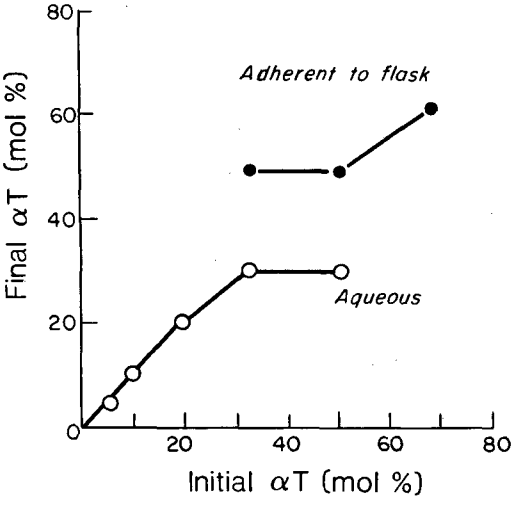

FIG. 2. The molar ratios of αT to PC are measured both in the aqueous phase (○) and in the film of dried lipid which adheres to the walls of the evaporating flask after the aqueous phase is removed (•). These values are compared to the initial molar ratios of αT in ethanolic solution.

tocopherol concentration of 67 mol %, all of the lipids were adherent and none entered the aqueous phase.

Regardless of the initial ratios used, no more than 30 mol % of αT ever entered the aqueous

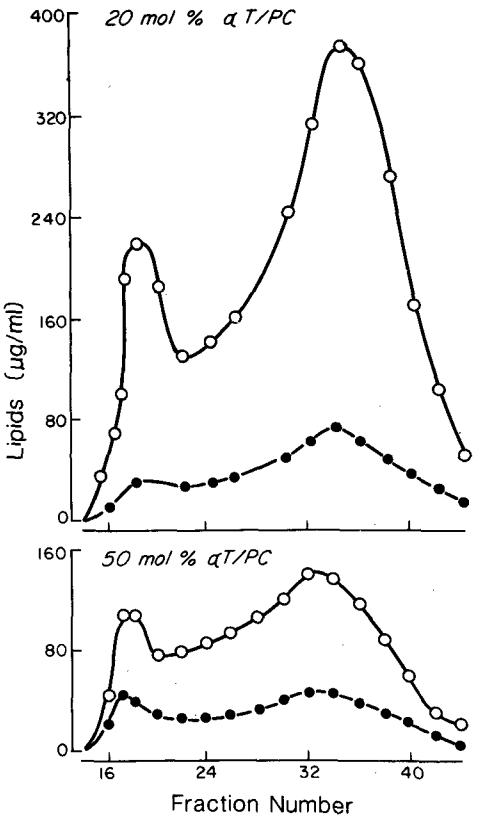

FIG. 3. Elution profiles from a 2% agarose column (1.2 × 95 cm). Each fraction contains 1.9 ml of elutant. Phospholipid (○) is measured as inorganic phosphorus and reported in μg/ml elutant. Tocopherol (•) is also reported in μg/ml elutant. Top: αT-PC liposomes prepared from a starting ratio of 20 mol % αT. Bottom: αT-PC liposomes prepared from a starting ratio of 50 mol % (aqueous dispersion contained 30 mol % αT; see Figure 2). These results are reproducible.

phase, thus putting an upper limit on the amount of tocopherol available for liposome formation. When initial concentrations of either 33 or 50 mol % αT were used, the amounts of αT and PC adhering to the glass maintained a strict 1:1 molar ratio (Fig. 2).

Liposomal Structure

The aqueous dispersions from two lipid mixtures (one with an initial tocopherol ratio of 20 mol %, and the other of 50 mol %) were chosen for liposome production and characterization studies. The aqueous dispersions were passed three times through a French pressure cell at 20,000 psi and then analyzed by column chromatography. Evaluation of the αT and PC content of the collected fractions (Fig. 3) in

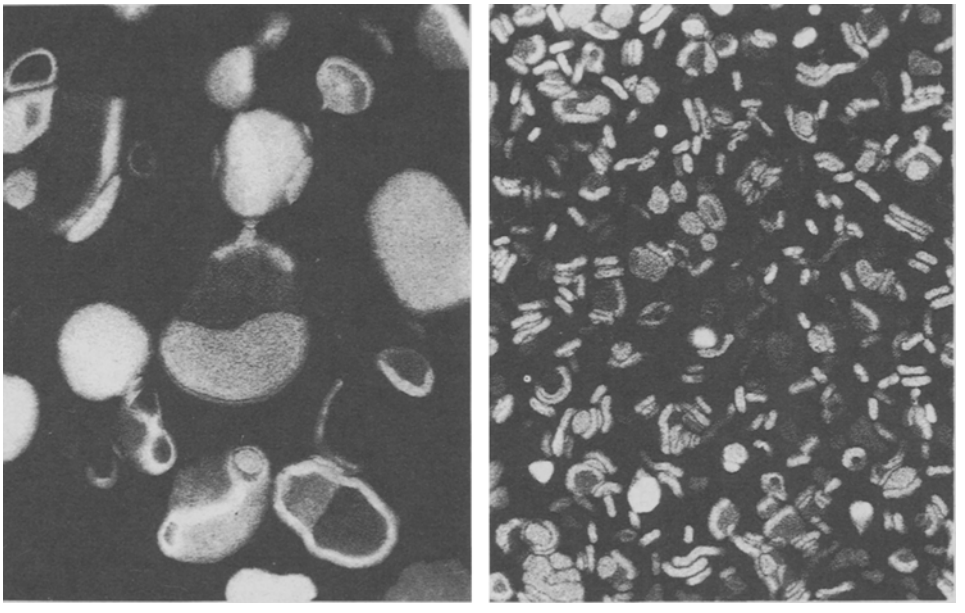

FIG. 4. Electron micrograph of negatively stained αT-PC (20 mol %) liposomes. Left: (× 180,000 Sample from the void volume shows pleomorphic multilamellar forms with diameters between 1000 and 6000 Å. Right: (× 180,000) Sample from the included volume peak fraction shows flattened unilamellar liposomes with an average long diameter of 280 Å. Only one type of particle is appreciated.

both cases showed that these two lipids co-eluted and maintained fairly constant molar ratios across the included volume peak. These ratios were 23 mol % αT when the aqueous dispersion contained 20 mol % tocopherol and 33 mol % when the aqueous dispersion contained 30 mol % tocopherol. Note the markedly reduced lipid recovery in the latter sample prepared from a 50 mol % ethanolic solution.

Electron micrographs of the material in the excluded volume peak from the 20 mol % preparation of liposomes (Fig. 4, left) show large, pleomorphic, multilamellar particles ranging in size from 1000 to 6000 Å. The included volume peak from the same sample (Fig. 4, right) shows smaller, more uniform particles, suggesting that the structures are small unilamellar liposomes. A mean particle diameter of about 280 Å was obtained by measuring the largest dimension of each flattened vesicle. This size is consistent with the presence of a single bilayer wall.

Stability of Liposomes at 37 C

CF-labeled PC liposomes containing αT at final molar concentrations of either 15 mol % or 29 mol % were prepared and compared to CF-containing liposomes composed of either pure PC or 37 mol % cholesterol. At 37 C all preparations showed a slow increase in dye release with time (Fig. 5). Pure PC liposomes released about 40% of their dye content within 2 hr. The 29 mol % tocopherol liposomes released about 10% of their dye content, while both the 15 mol % tocopherol and the 37 mol % cholesterol liposomes released only 5% after 2 hr.

Stability Following Exposure to HDL Apoprotein

When exposed to apo-HDL (50 μg in 1 ml column buffer) the pure PC liposomes rapidly released dye (60% release within 2 min) (Fig. 6, top). Cholesterol-containing liposomes showed

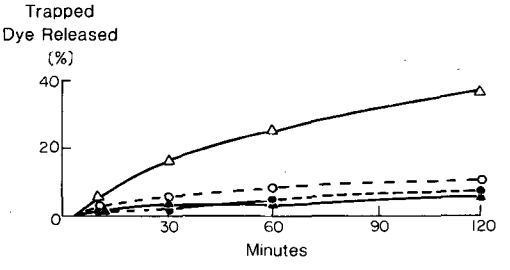

FIG. 5. The release of CF from non-chromatographed liposomes is measured at 37 C. The percent release from pure egg PC (△) is compared to release from liposomes containing 37 mol % cholesterol (▲), 15 mol % αT (●) and 29 mol % αT (○). The data shown are from one experiment. Repeat studies showed similar curves.

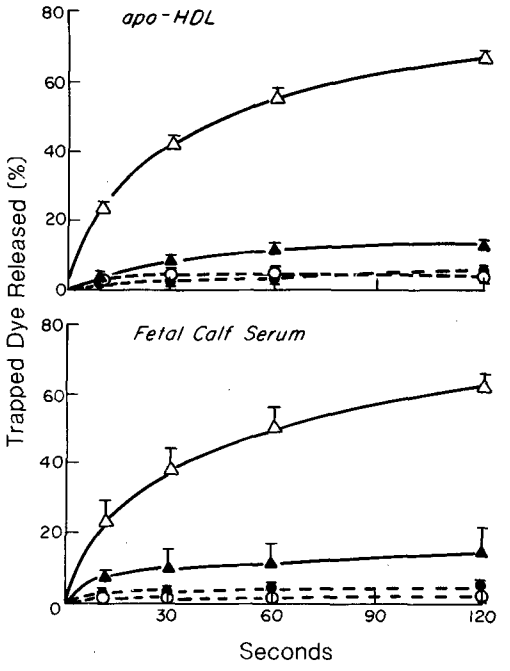

FIG. 6. The percent release of CF dye after 10 μl liposomes are mixed with 1 ml of protein-containing buffer at 37 C. Egg PC (△); 37 mol % cholesterol (▲); 15 mol % αT (●); and 29 mol % αT (○). Top: Release of CF from liposomes after incubation with apo HDL (50 μg/ml buffer). Bottom: Release of CF from liposomes after incubation with 15% fetal calf serum in buffer. These curves were constructed from one experiment. Repeat studies showed similar patterns.

greater stability (13% dye release at 2 min), but the tocopherol-containing preparations were the most stable, releasing less than 5% of their trapped dye on exposure to apo-HDL.

Stability After Exposure to Fetal Calf Serum

Similar differences in liposome stability also were demonstrated by incubation with 15% FCS in buffer (Fig. 6, bottom). We studied the interaction of CF dye (10^{-9} to 10^{-5} M) with 15% FCS solutions and found that, although the fluorescent intensity was decreased by about 15% as compared to buffer alone, the fluorescent intensity was still linear within this range. Also, there was no evidence of quenching upon the addition of a 20% Triton solution (1:10, v/v).

Stability After Storage at 4 C

After 6 weeks of storage in an argon atmosphere at 4 C, the liposomes were again examined for dye release at 37 C. The amount of background fluorescence, F_B, found immediately after warming to 37 C was determined according to Equation [2]. The pure PC liposomes (Table 1) showed a marked increase in F_B as evidenced by a loss of 43% of their trapped dye. The liposomes containing 37 mol % cholesterol lost 28% of their dye after storage. In contrast, the 15 mol % and 29 mol % tocopherol liposome preparations had lost only 2.3% and 3.1% of their trapped dye, respectively. The changes seen in the αT-containing liposomes were lower (both in % change from Day 0 and in absolute value) than the changes seen with either the pure PC or cholesterol-containing liposomes.

DISCUSSION

These studies show that liposomes of egg PC can incorporate up to 33 mol % of alpha tocopherol (Fig. 3) when prepared using a French pressure cell. Our data regarding tocopherol concentration in PC vesicles differs from the findings of Bellmare and Fragata (8), who reported a maximum molar ratio of 4.8 mol % alpha tocopherol in their liposome preparations prepared by sonication. This difference could be due to the method of preparation, to differences in the % of unsaturated acyl chains in the egg PC preparations (as suggested by Diplock et al. [9]), or to the method of measuring tocopherol content. We used an assay based on the direct fluorescence of tocopherol after the liposomes had been disrupted and the lipids saponified and extracted into hexane. All of our lipids were quantitatively recovered. Bellmare and Fragata used an indirect fluorescent assay based on the interaction of tocopherol with the stable free radical, 1,1-diphenyl-2-picrylhydrazine (DPPH). Furthermore, some of their measurements were performed on intact liposomes in dilute ethanolic solution. Our experience has been that measurements of the fluorescence of tocopherol in intact liposomes is not linear, possibly because geometrical constraints lead to quenching. Also, it may be that differences in the oxidation state of tocopherol can affect the DPPH assay more severely than the direct fluorometric assay.

Tocopherol-containing liposomes are larger than those of egg PC alone when formed by our procedure. This increase in vesicular size is similar to that seen when cholesterol is incorporated into PC liposomes. Several other properties of the tocopherol-containing liposomes are also similar to those of unilamellar vesicles containing a comparable proportion of cholesterol. First, the diffusion of an entrapped water soluble dye, carboxyfluorescein, is markedly retarded by the presence of alpha

tocopherol in the bilayer. Secondly, the disruption of unilamellar liposomes by fetal calf serum and serum apolipoproteins is largely impeded by the incorporation of tocopherol into the vesicles. The membrane stabilizing effect of alpha tocopherol with respect to protein-induced disruption was found to be more pronounced than that of cholesterol, even with a lower molar ratio of tocopherol. Liposomes containing alpha tocopherol also appear to remain intact and relatively non-permeable at 4 C for up to 6 weeks, as shown by the very small loss of CF dye (Table 1). The large loss of dye exhibited by the pure egg PC liposomes probably reflects a slow outward diffusion of CF because previous studies (11) have shown that these liposomes remained structurally intact over long periods of storage.

Unilamellar liposomes containing between 15 and 30 mol % of alpha tocopherol may have value as carriers of physiologic amounts of tocopherol in biological systems. It has been shown (18) that phosphatidylcholine liposomes containing 20 mol % of tocopherol increase neuronal lifespan and decrease gliosis in an in vitro, neuro-glial culture system. Furthermore, these tocopherol-phospholipid liposomes may have potential for the treatment of patients with severe vitamin E deficiency secondary to lipid malabsorption states.

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COMMUNICATIONS

Lipid Composition of Different Areas of Murine Brain: Effects of Lipid Extraction Procedures

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ABSTRACT

The effects of various chemical extraction procedures on the determination of lipid composition of rat and mouse brain have been investigated. Tissue extractions with formic acid/acetone or perchloric acid both resulted in significant losses of total phospholipids and cholesterol. Perchloric acid extraction also degraded, almost quantitatively, ethanolamine plasmalogens to lysophosphatidylethanolamine. Our findings have thus demonstrated that conventional procedures used for extraction of brain tissue for analysis of choline and acetylcholine content cannot also be used for concurrent/simultaneous extraction of phospholipids and cholesterol from the same tissue.
Lipids 20:201-203, 1985.

INTRODUCTION

Our laboratory currently is studying the effects of various pharmacologic agents on cholinergic neurotransmission, as well as on the structure and function of brain cells and cellular membranes. For such studies a method of lipid extraction is required which yields complete extraction of choline (Ch) and acetylcholine (ACh) while excluding or minimizing their degradation.

Previous work from this laboratory (1,2) has stressed the need for using acid extraction procedures for the quantitative isolation of Ch and ACh from brain tissue. The most commonly used procedures to extract lipids involve the use of mixtures of chloroform-methanol. However, the suitability of such mixtures in conjunction with the extraction of Ch and ACh has not been examined.

In this publication, we report on studies in which we have compared different conventional methods of extraction of Ch and ACh, and have studied their effects on the determination of phospholipid composition in different areas of the rat and mouse brain.

MATERIALS AND METHODS

Animals. Male and female rats (Sprague Dawley strain, Zivic Miller Laboratories, Allison Park, Pennsylvania) and female mice (CD-1 strain, Charles River Laboratories, Wilmington, Massachusetts) were killed by decapitation. Whole brain was taken, or hippocampus, cortex and striatum, were dissected from each side of the brain, and tissue was weighed and processed as described in the following sections.

Extraction of choline and acetylcholine. Brain tissue was homogenized for 20 sec in 0.4N $HClO_4$ or formic acid (1N)/acetone (85/15; v/v). Homogenates were centrifuged at 16,000 r.p.m. (Sorval centrifuge, rotor SM24) for 20 min. The supernatants were used for the quantitation of Ch and ACh according to published procedures (1,2). The pellets were stored frozen at −60 C, until subjected to lipid extraction and analysis (see below).

Lipid extraction. Lipid extraction was performed according to Bligh and Dyer (3). Tissue or the pellet (obtained after the extraction of Ch and ACh) was homogenized in chloroform:methanol (2:1, v/v). The homogenate was filtered through Whatman #43 filter paper. The resulting clear filtrate was shaken with 0.9% NaCl, and the layers allowed to separate overnight at 4 C. The lower layer was removed, evaporated to dryness under N_2, and reconstituted in $CHCl_3$. The lipids extracted into $CHCl_3$ were subjected to total cholesterol, as well as total and individual phospholipid quantitation.

Cholesterol was estimated by the method described by Bowman and Wolf (4). Total phospholipid P was estimated by the method of Shin (5).

Individual phospholipids were separated by a two dimensional thin layer chromatographic procedure, detailed by Katyal and Lombardi (6). The plates were chromatographed in the first dimension in chloroform:methanol:ammonium hydroxide (58%) (65:35:5, v/v/v) and in the second in n-butanol:acetic acid:H_2O (90:20:20, v/v/v). Spots were visualized by exposure to I_2 vapors. Spots were scraped and

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eluted with a mixture of chloroform-methanol-acetic acid-H_2O (50:30:1:10, v/v/v/v). Aliquots of the eluted lipids were subjected to a quantitation of phospholipid P (5).

The plasmalogen content of ethanolamine phosphatides was quantitated by the method described by Horrocks (7). In this approach plasmalogens are cleaved into free aldehyde and lysophosphatidylethanolamine. Spots of lyso- and phosphatidylethanolamine are scraped, eluted and the eluate used for P determination as described above.

RESULTS AND DISCUSSION

Among the various procedures for the extraction of Ch and ACh, those involving homogenization of the tissue in formic acid/acetone and perchloric acid are most commonly used (1,2). The clear supernatant obtained after centrifugation of the homogenates is used for the quantitation of Ch and ACh. In the present study, the lipids all were extracted with chloroform-methanol from the pellets obtained from these different types of homogenates. Total cholesterol, as well as total and individual phospholipids in different areas of the mouse brain, were determined.

The results obtained from direct extraction of cortex with chloroform-methanol were compared with those obtained from pellets obtained following homogenization of tissue in either formic acid/acetone or perchloric acid, respectively (Table 1). It is evident that significant losses of total phospholipids and cholesterol occurred during the treatment of cortex either with perchloric acid (Method 2) or with formic acid/acetone (Method 3).

The content of ethanolamine phospholipids showed about a 50% reduction as a result of tissue extraction with perchloric acid (Table 1, compare methods 1 and 2). This reduction can be accounted for by the simultaneous appearance of lysophosphatidylethanolamine (Figure 1b, spot 7), the contents of which rose to about 18% of the total phospholipid phosphorous (Table 1). Lysophosphatidylethanolamine was barely detectable when lipids were extracted by method 1 (Fig. 1a).

Perchloric acid extraction thus appears to result in almost complete degradation of ethanolamine plasmalogens to lysophosphatidylethanolamine, presumably due to susceptibility of the former to acidic conditions. In a separate experiment, ethanolamine plasmalogens were quantitated and accounted for about 50% of the total ethanolamine phospholipids. On the other hand, no significant loss of choline phospholipids occurred as a result of perchloric acid extraction. Evidently this is due to the reported low content of choline plasmalogens in brain tissue (8). Interestingly, a minor, but significant, increase in the content of lysophosphatidylethanolamine also was observed as a result of tissue extraction with formic acid/acetone (Table 1). Lipid analyses of other areas of the brain after extractions with each of the 3 methods showed similar results (data not presented).

Our findings have demonstrated that conventional procedures used for extraction of brain tissue samples for analysis of Ch and ACh con-

TABLE 1

Distribution of Lipids in the Cortex Region of Mouse Brain. Comparison of Different Methods of Extraction of Lipids.

	Method 1	Method 2	Method 3
Total cholesterol (mg/g brain)	17.3 ± 0.7(4)	12.5 ± 0.5(8)	5.4 ± 0.6(5)
Total phospholipids (mg/g brain)	52.2 ± 1.2(4)	44.3 ± 0.7(8)	33.5 ± 0.7(7)
Individual phospholipids (percent total phospholipid P)			
Sphingomyelin	3.6 ± 0.1(4)	4.2 ± 0.2(5)	5.0 ± 0.1(6)
Choline phospholipids	40.4 ± 0.2(4)	41.3 ± 0.6(5)	36.6 ± 1.0(6)
Ethanolamine phospholipids	37.3 ± 0.3(4)	19.1 ± 0.9(5)	34.0 ± 0.8(6)
Phosphatidylinositol + phosphatidylserine	16.5 ± 0.2(4)	17.3 ± 0.4(5)	20.6 ± 0.5(6)
Others	2.0 ± 0.0(4)	18.0 ± 0.3(5)[a]	4.5 ± 0.3(6)[a]

Method 1: Lipids were extracted by homogenization of mouse brain tissue in chloroform/methanol (2:1, v/v).

Method 2: Lipids were extracted with chloroform:methanol (2:1, v/v) from pellets obtained from tissue homogenates in perchloric acid.

Method 3: Lipids were extracted with chloroform:methanol (2:1, v/v) from pellets obtained from tissue homogenates in formic acid/acetone.

[a]Includes lysophosphatidylethanolamine, with relatively small amounts of phosphatidic acid.

Values are mean ± S.E. of the number of animals given in parentheses.

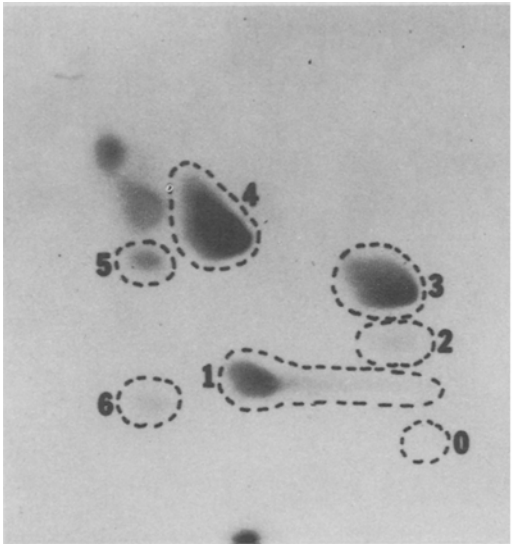

FIG. 1a. Two dimensional thin layer chromatographic separation of lipids extracted from brain tissue homogenized in chloroform-methanol (2:1, v/v). Lipids were spotted at the bottom right hand position of the thin-layer plate and chromatographed in the first dimension in chloroform-methanol-NH_4OH (58%) 65:35:5, v/v/v. After removal from the solvent, the plates were chromatographed in the second dimension in n-butanol-acetic acid-H_2O 90:20:20, v/v/v. Spots were located by exposure of the plate to iodine vapors and photographed. Phosphorus-containing spots are: (0), origin; (1) phosphatidylserine, phosphatidylinositol plus lysophosphatidylcholine; (2) sphingomyelin; (3) choline phospholipids; (4) ethanolamine phospholipids; (5) cardiolipin and (6) phosphatidic acid. Unidentified spots represent substances that do not contain phosphorus.

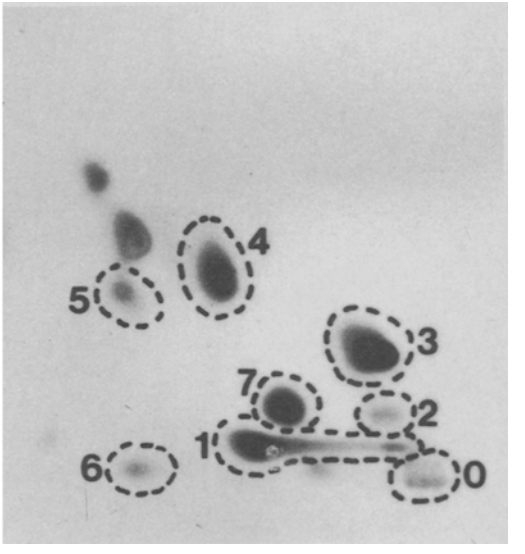

FIG. 1b. Two dimensional thin layer chromatographic separation of lipids extracted from brain tissue homogenized in 0.4N perchloric acid. For other details see legend to Figure 1a. Note the presence of an extra spot (#7) which represents lysophosphatidylethanolamine.

tent cannot also be used for the concurrent/simultaneous extraction of phospholipids and cholesterol from the same tissue. The acidic conditions, used primarily for the purpose of maintaining the stability of extracted ACh in the medium, create qualitative and quantitative alterations in the lipids. Considerable amounts of lipids are lost in the supernatants after acid precipitation, and/or are destroyed by the acidic conditions. Thus, any attempt to interrelate levels of Ch and ACh, on the one hand, and measures of various phospholipids and cholesterol on the other, in the brains of the same animals following acid extraction of these substances, must take these observations into account.

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Fatty Acid Synthesis in the Oil Palm *(Elaeis guineensis)*: Incorporation of Acetate by Tissue Slices of the Developing Fruit

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ABSTRACT

Oil palm *(E. guineensis)* fruits at three stages of development were studied. At week 12-13 after anthesis, the endosperm had started accumulating oil and tissue slices incorporated [1-^{14}C] acetate into fatty acids which resembled those found in the mature endosperm. The mesocarp contained very little oil and incorporated acetate into polar lipids. At week 16-17, the mesocarp started to accumulate oil; this was reflected in the [^{14}C] lipid products from acetate incubation. At or just prior to this stage, an increase in the endogenous linoleic and linolenic acid content and the increase in fruit size indicated cellular growth in the mesocarp tissue. At week 20-21 the fruit was ripe, and both endosperm and mesocarp tissues were filled with storage oil. [^{14}C] Fatty acids synthesized from acetate by mesocarp slices at this stage were the same as the endogenous storage fatty acids in both *E. guineensis* and *E. oleifera*. A very weak fatty acid synthesizing activity was seen in the mature endosperm, but the products had no relationship to the storage lipid.
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INTRODUCTION

The fruit of the oil palm *(Elaeis guineensis)* is the source of two commercial edible oils. The endosperm (kernel) produces an oil which resembles coconut oil in fatty acid composition and contains 60-70% lauric and myristic acids (1-3). The fatty acid composition of the mesocarp oil shows an average of 44% palmitic, 39% oleic and 10% linoleic acids (3-5). In the Central American oil palm, *Elaeis oleifera*, the mesocarp oil shows a higher degree of unsaturation (17% palmitic, 60% oleic and 19% linoleic acids), while *E. oleifera* x *E. guineensis* hybrid palms produce mesocarp oils with intermediate levels of oleic and linoleic acids (4). The fruit is therefore a very interesting tissue to study fatty acid biosynthesis.

The long-term objective of this research is to study the regulation of fatty acid biosynthesis in the oil palm fruit with respect to the variation in fatty acid composition of the mesocarp oil from *E. guineensis*, *E. oleifera* and their hybrids as well as the possible mechanism of chain termination. To meet this objective, information concerning the pathways of fatty acid and acylglycerol biosynthesis in the tissue is needed. This paper reports the use of radioactive acetate to follow fatty acid and lipid synthesis in tissue slice preparations. The aim is to define the stage of fruit development when active fatty acid synthesis in the mesocarp and endosperm incorporate products reflective of the storage lipids in those tissues. This information is important for the choice of material to study cell-free systems of fatty acid biosynthesis in the oil palm.

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MATERIALS AND METHODS

[1-^{14}C] Acetic acid, sodium salt (57 Ci/mol) was obtained from Amersham International, England. Ten percent EGSS-X on Gas Chrom Q (100/200 mesh) was from Supelco, Bellefonte, Pennsylvania. Fruit spikelets at stages of development ca. 12-13 weeks, 16-17 weeks and 20-21 weeks after anthesis were obtained fresh from the experimental station of PORIM (Palm Oil Research Institute of Malaysia, Bangi, Selangor, Malaysia) and used the same day. The fruits were chosen from tenera (D x P) plantings.

Tissue Slice Incubations

The individual fruits were separated from the spikelet and washed with tap water to remove dirt. The thin exocarp was sliced off carefully. Slices (1 mm thick) of the underlying yellowish mesocarp were cut with a razor blade. These were then rinsed with 3 x 10 ml of 0.1 M tricine-NaOH buffer (pH 7.5) to remove endogenous lipids from the cut surfaces. Excess buffer was blotted off with filter paper, and 1g samples were weighed for incubation. With *E. oleifera* fruits it was not possible to make good slices from the thin mesocarp layer. Instead the mesocarp layer (1.5-2 mm thick) was simply peeled away from the nut, rinsed and weighed. To prepare endosperm slices, the fruit was split into two with a sharp knife. The endosperm was scooped out and slices were made with a razor blade. The tissue slices (1g) were incubated with 3 ml of solution containing 0.1M tricine-NaOH buffer (pH 7.5), 0.025M $KHCO_3$ and 15μCi [1-^{14}C] sodium acetate (to obtain fatty acid products of sufficiently high specific radioactiv-

ity for analysis). The incubation was carried out in a 25ml conical flask capped with a rubber stopper carrying a plastic center well (Kontes Sc. Glassware, Vineland, New Jersey). A small piece of fluted filter paper moistened with 0.2ml of 2M KOH was placed in the well to absorb $^{14}CO_2$, and the flask was gently shaken for 4 hr at ambient temperature (27 C).

Analysis of Tissue Slice Incorporation Products

At the end of the incubation period, 0.2ml of 4M H_2SO_4 and 0.3ml of 1M sodium acetate solution were added and the flask was shaken for 10 min. The moist filter paper was transferred directly into a scintillation vial for counting. The supernatant was removed and the tissue slices were extracted with 3 x 15ml of chloroform/methanol (2:1, v/v) overnight, followed by two more extractions the next day. The combined extract was washed with 0.2 volume of 0.8% NaCl solution. The chloroform layer was concentrated on a rotary evaporator under reduced pressure and then evaporated to dryness under nitrogen. The residue was redissolved in 4ml of hexane. The reaction mixture supernatant was combined with the slice washing and extracted with 3 x 2ml of hexane. The [^{14}C] lipids extracted from this mixture usually was less than 0.5% of the total [^{14}C] lipids formed. Samples of lipid extracted from the slices and the reaction mixture supernatant as well as samples of the aqueous layers from the extractions were counted in 10 ml of scintillation fluid made up of 2 l toluene, 1 l triton X-100, 8g PPO and 0.2g dimethylPOPOP. Radioactive counting was carried out on a Packard Tricarb 3255 liquid scintillation counter.

Analysis of Lipid Classes

Lipid extracts were separated into mono-, di- and triacylglycerols, free fatty acids and polar lipids by chromatography on silica gel G plates using the solvent system hexane/diethylether/acetic acid, (80:20:2, v/v/v) (6). Radioactive lipid spots located by exposure to iodine vapor were scraped into liquid scintillation vials and counted. For analysis of the fatty acid components of these lipid classes, the chromatogram was run in streaks. The lipid bands were recovered using a TLC sample recovery tube (Kontes Sc. Glassware, Vineland, New Jersey) and directly eluted with chloroform/methanol (2:1, v/v) solvent.

Analysis of Fatty Acid Composition

Lipid samples were saponified with 0.3 ml of 8M KOH and 1 ml of 50% methanol for 40 min at 80 C. The mixture was then acidified with 0.2 ml of 6M H_2SO_4 and extracted with 2 x 2 ml of hexane. The extract was reduced to about 0.5 ml under nitrogen and methylated with an excess of freshly prepared ethereal solution of diazomethane.

Radioactive fatty acid methyl esters were analyzed on a Hewlett-Packard 5790 Gas Chromatograph with a FID detector and coupled to a Packard 894 Gas Proportional Counter by an effluent splitter. Routine analysis was carried out at 170 C on a 6ft x ¼in. stainless steel column packed with 10% EGSS-X on Gas Chrom Q using argon as carrier gas and propane as quencher gas. The mass traces of the fatty acids from endogenous lipids of the tissue slices were quantitated with a Hewlett-Packard 3390A Integrator.

RESULTS AND DISCUSSION

Stage of Fruit Development

Fruit development and oil deposition in the oil palm were described by Hartley (3) and Thomas et al. (7). The oil palm fruit is a drupe which grows in a tight bunch (3). Each mature fruit contains a nut consisting of a hard woody endocarp (shell) enclosing the endosperm (kernel). Surrounding the nut is the fibrous oily mesocarp (pulp) covered by a thin smooth skin (exocarp). Fruit development and differentiation starts ca. two weeks after anthesis (7). At week 8 the endosperm content of the seed is still liquid, but it turns semi-gelatinous at week 10 (3,8). Oil deposition in the endosperm starts at ca. week 12 and is almost complete by week 16 (8). During this period the endosperm and the endocarp slowly harden. At ca. week 16-17, while there is still little oil in the mesocarp, the endosperm is a whitish hard tissue enclosed by a hard woody shell. Oil deposition in the mesocarp starts at ca. week 15 and continues until fruit maturity at ca. week 20-21. Almost all the mesocarp oil is deposited between week 19-20 in Nigerian palms (9), but in Malaysian plantations oil deposition occurs more evenly (7).

It is difficult to define precisely the age of the fruits used in the present study since the flowers were not hand-pollinated. However, three stages of development were used based on the following criteria. The first stage (Stage A) was when oil deposition was actively occurring in the endosperm. The fruit was still small, greenish-yellow in color and black at the tip. It was easily sliced in two with a knife. Inside, the endocarp was cream-colored and fully formed but not hard. The endosperm was firm but also not hard. The estimated age was week 12-13 after anthesis (between stages E and F as de-

TABLE 1

Fatty Acid Composition of Endogenous Lipids of Fruit Mesocarp and Endosperm Tissues[a]

Tissue	Stage of development[b]	Total endogenous lipid (g/g tissue)	Percentage of total fatty acids								
			8:0	10:0	12:0	14:0	16:0	18:0	18:1	18:2	18:3
Mesocarp	A	0.01	–	–	0.2	0.6	36.5	1.9	40.9	18.5	1.4
	B	0.02	–	–	2.4	1.6	36.5	2.3	18.5	28.5	10.4
	C	0.31	–	–	0.2	1.0	40.3	2.5	43.2	12.8	–
	C[c]	0.15	–	–	tr	0.1	20.4	tr	51.3	26.0	2.2
Endosperm	A	0.03	0.9	1.2	25.0	12.8	12.6	1.6	36.7	9.2	–
	C	0.32	3.5	3.8	48.3	19.9	8.1	1.9	12.0	2.1	–

[a]Endogenous lipids were extracted from the same slice preparations used in acetate incubations. Procedures for lipid extraction and estimation of fatty acid composition are described in the Methods section.

[b]For estimate of stage of development, see Results section.

[c]Mesocarp of *E. oleifera* fruit.

scribed by Thomas et al.) (7). The second stage of development chosen (Stage B) was when oil deposition had finished in the endosperm and started in the mesocarp, i.e., ca. week 16-17 (7,8). In practical terms the endocarp was deep brown in color, hard, and required a sharp knock with a sharp knife to cut through. The mesocarp was greenish-yellow and non-oily, and the exocarp was deep red at the outer tip with a developing orange color toward the stalk. The third stage (Stage C) was at fruit maturity. Mature fruits were obtained from fruit bunches which were harvested according to normal commercial practice, i.e., when a certain percentage of fruits on the bunch became loose (10). These fruits were at week 20-21 and were bright waxy orange in color, while their mesocarp was bright yellow and filled with oil (7).

Endogenous Fatty Acid Composition

Table 1 shows the lipid content of fresh mesocarp and endosperm tissues and the fatty acid composition of these lipids at different stages of fruit development. There was a two-fold increase in the lipid content of mesocarp tissue between Stage A and Stage B. While this indicated that oil deposition in the mesocarp had started at Stage B, not all the increase could be attributed to storage lipid. Between Stage A and Stage B fresh tissue apparently was still being synthesized, because a 9% increase in fresh fruit weight was reported (7). Between Stage B and Stage C, however, there was no further increase in fruit weight, and the 15-fold increase in lipid content of the mesocarp tissue (Table 1) all could be attributed to storage lipid. By comparison with *E. guineensis*, Stage C (mature fruit) mesocarp of *E. oleifera* contained only half as much oil. Similar observations were reported by Meunier and Hardon (11) viz. 30% oil in fresh mesocarp of *E. oleifera* compared to 50% in *E. guineensis*. In the endosperm, there was a 10-fold increase in lipid content between Stage A and Stage C. Most of this oil was deposited within 2-3 weeks after Stage A (8).

The fatty acid compositions of Stage C tissues were the same as those of commercial palm oil (4) and palm kernel oil (3), because the fruits were harvested according to commercial criteria for ripeness. The lipids in Stage C fruits were, therefore, mainly storage oils. This was not the case at earlier stages of development, as can be seen from the fatty acid compositions. In the mesocarp, larger proportions of 18:2 and 18:3 acids were present in younger fruits, reflecting the higher ratio of cellular to storage lipids in these fruits. The occurrence of a fairly large amount of linolenic acid at Stage B which disappeared from the 20-week mesocarp also was reported by Crombie (9). It probably was diluted below detectable levels in lipid extracts by the massive accumulation of storage lipids at week 20 which contained very little linolenic acid. Tan et al. (12) separated palm oil by silver nitrate TLC. Only 5.09% of the triacylglycerols were found to contain four or more double bonds, and of this fraction only 4.9% was linolenic acid. The increase in 18:2 and 18:3 acids between Stage A and Stage B mesocarp might be a reflection of tissue growth. During this period there was a 9% increase in fruit size (7), and it is not inconceivable that as oil deposition in the endosperm was completed, it was accompanied by a short period of cellular proliferation in the mesocarp in anticipation of oil deposition in that tissue. Crombie (9) found only negligible amounts of oleic acid in mesocarp oil before week 19 in contrast to the present results (Table 1). This might be related to the fact that in their study, almost all the mesocarp oil was

TABLE 2

Incorporation of [1-^{14}C] Acetate into Fatty Acids by Mesocarp and Endosperm Tissue Slices from Oil Palm Fruit[a]

Tissue	Stage of development[b]	nmoles acetate incorporated into fatty acids	Distribution of [^{14}C] into fatty acids (%)							
			8:0	10:0	12:0	14:0	16:0	18:0	18:1	18:2
Mesocarp	A	9.56	–	1.0	1.2	1.2	17.1	3.1	73.0	2.6
	B	11.37	–	1.0	1.3	1.1	25.0	2.2	61.3	8.1
	C	23.77	–	1.2	3.0	2.8	36.9	2.0	52.6	1.5
	C[c]	23.25	–	–	–	–	22.4	–	67.6	10.0
Endosperm	A	1.42	0.5	12.5	48.2	25.4	7.4	–	6.0	–
	C	0.19	–	3.4	18.0	4.8	29.0	20.1	18.5	6.2

[a]Tissue slices (1g) were incubated with 258 nmoles of [1-^{14}C] acetate and the lipid products extracted and analyzed as described in the Methods section. Fatty acid compositions of the endogenous lipids of the slices are given in Table 1.

[b]For estimate of stage of development, see Results section.

[c]Mesocarp of *E. oleifera* fruit.

deposited in week 19-20 while the material used here showed a more even deposition of oil in the mesocarp starting from ca. week 15 (7).

In the endosperm the proportion of 12:0 and 14:0 acids at Stage A already was high compared to their final values at Stage C. This implied that in the Stage A endosperm tissues used in this study, active synthesis of storage lipids was taking place (Table 2) even though it had deposited only 10% of its final storage oil.

Effect of Stage of Development on Incorporation of [1-^{14}C] Acetate into Fatty Acids by Tissue Slices

The use of radioactive acetate as a substrate for in vivo incorporation into lipid products by a variety of plant tissues is a routine procedure. Recently lipid synthesis in an oil palm tissue culture using [1-^{14}C] acetate was studied (13). The experimental material used were oil palm callus in the process of differentiation into embryoids. It was therefore different from the specialized, mature fruit tissues employed here. Nevertheless, acetate was shown to be incorporated mainly into polar lipids during cell division associated with differentiation into embryoid tissue. Subsequently the embryoids synthesized both triacylglycerol and polar lipids from the acetate substrate. Unfortunately the compositions of the [^{14}C] fatty acids in these lipid products were not studied.

The synthesis of [^{14}C] fatty acids from [1-^{14}C] acetate by mesocarp and endosperm tissue slices is shown in Table 2. In the mesocarp it is obvious that this activity was highest at Stage C in line with the increase in oil deposition at this stage (7,9). Since Stage C slices contained considerably more endogenous lipid than Stage A or Stage B slices, the activity relative to that of Stage A and Stage B would be even greater than the values shown in Table 2, if expressed as nmoles acetate incorporated per g non-fat dry weight of tissue. Most of the [^{14}C] fatty acid synthesized from [1-^{14}C] acetate by Stage C mesocarp slices is expected to be incorporated into storage lipid. This was seen in the composition of the [^{14}C] fatty acids synthesized which resembled the endogenous fatty acids in both *E. guineensis* and *E. oleifera* Stage C mesocarp. However, during the 4-hr incubation both tissues synthesized more [^{14}C] oleic acid and less [^{14}C] linoleic acid than present in their endogenous lipid (Table 1). Linoleic acid is a constituent of palm oil triacylglycerols (12) making up 9-11% of the commercial oil (4). It is therefore apparent that the de novo synthesis of oleic acid from acetate occurred much more rapidly than the subsequent desaturation to linoleic acid.

The [^{14}C] fatty acids synthesized by Stage A and Stage B mesocarp slices were different from their endogenous fatty acids. Less [^{14}C] palmitic acid and more [^{14}C] oleic acid were synthesized than at Stage C, and the difference was greater the younger the fruit. Stage A mesocarp contained no storage lipid (7,9), and lipid synthesis at this stage was associated with membrane and cellular lipids. This was supported by the fact that 81.6% of the [1-^{14}C] acetate incorporated was found as polar lipids (Table 3). At Stage B, oil deposition in the mesocarp had started (Table 1). This was reflected in the lower percentage (41%) of [^{14}C] polar lipids synthesized. Although polar lipids are final products in membrane synthesis, they are involved as intermediates in the formation of triacylglycerols in oil seeds (14-17). It is therefore not surprising to note that ca. 20% of the radioactivity incorporated by Stage C mesocarp was located in

TABLE 3

Formation of [^{14}C] Acylglycerols from [1-^{14}C] Acetate by Mesocarp and Endosperm Tissue Slices from Oil Palm Fruit[a]

Tissue	Stage of development[b]	Distribution of [^{14}C] incorporated (%)[c] TG	DG	MG	FA	PL
Mesocarp	A	7.8	7.2	2.0	2.2	81.6
	B	33.0	14.4	4.6	7.0	41.0
	C	23.6	34.0	3.8	17.9	20.8
	C[d]	20.0	51.2	0.8	1.4	26.7
Endosperm	A	41.0	9.9	0.9	36.5	11.7
	C	17.8	40.8	6.5	18.8	16.1

[a]Tissue slices (1g) were incubated with 258 nmoles of [1-^{14}C] acetate, and the lipid products were extracted and separated into lipid classes by TLC as described in Methods section.

[b]For estimate of stage of development, see Results section.

[c]TG, DG, MG = tri-, di-, monoacylglycerol; FA = free fatty acid; PL = polar lipid.

[d]Mesocarp of *E. oleifera* fruit.

TABLE 4

Distribution of [^{14}C] Fatty Acids in Different Lipid Classes Obtained from Incubation of [1-^{14}C] Acetate with Mesocarp Tissue Slices[a]

Lipid class	Percentage of [^{14}C] incorporated 10:0	12:0	14:0	16:0	18:0	18:1	18:2
Total lipids	1.0	3.0	2.8	36.9	2.0	52.6	1.5
TG	–	4.1	6.3	30.5	4.8	50.1	3.9
DG	–	–	1.3	35.8	6.5	53.2	3.2
MG	–	2.1	1.1	44.4	4.1	43.4	4.1
FA	–	2.2	3.6	34.0	5.0	55.2	–
PL	–	–	0.5	48.1	5.4	40.5	5.6

[a]Mesocarp slices of Stage C *E. guineensis* fruits were used.

polar lipids (Table 3).

The fairly high level of free fatty acid among the [^{14}C] lipid products in some of the incubations deserves comment. It is unlikely that they represent final products of acetate incorporation. Their presence at levels of 18-36% (Table 3) might be due to lipolytic activity on acylglycerol products. Increased free fatty acid level as a result of bruising or tissue damage in ripe oil palm fruits has been reported (3,18), and Oo (19) has shown the rapid hydrolysis of endogenous triacylglycerols in ripe oil palm mesocarp slices. In the case of Stage A endosperm slices (Table 3), ca. 9% of the total [^{14}C] lipid products was recovered from the incubation solution and slice washing (see Methods). Analysis of the [^{14}C] fatty acids present in the different classes of [^{14}C] lipid products did not show any striking differences between them (Table 4). Stymne et al. demonstrated a rapid fatty acyl exchange between phosphatidylcholine and oleoyl-CoA, an interconversion between phosphatidylcholine and diacylglycerol and a rapid acylation of diacylglycerol to triacylglycerol by microsomal preparations of developing sunflower and safflower cotyledons (14,15). However, these reactions were not detected in avocado mesocarp (14) which, like oil palm mesocarp, is rich in oleate.

The incorporation of [1-^{14}C] acetate into [^{14}C] fatty acids by endosperm tissue slices resembled that found with coconut endosperm by Oo and Stumpf (2). As with the coconut, fatty acid synthesizing activity was observed when the oil palm fruit endosperm had solidified from a semi-gelatinous state into a firm tissue (Stage A). The activity, however, was considerably lower than that found in the mesocarp (Table 3). Nevertheless, the [^{14}C] fatty acids synthesized from [1-^{14}C] acetate resembled the endogenous fatty acids in the mature (Stage C) endosperm (Table 1), indicating that they were storage lipid constituents. A very weak fatty acid synthesizing activity was found in mature (Stage C) endosperm as was previously shown in the coconut. In both cases the products were mainly palmitic, stearic and oleic acids. The metabolic role of this synthesis is unclear, and the distribution of the [^{14}C] fatty acid residues in the lipid classes (Table 3) gave no further enlightenment.

In summary, it was established that active synthesis of storage lipids in the oil palm fruit was ca. week 12-13 for endosperm tissue and ca. week 20-21 for mesocarp tissue. At other stages of development, lipid products used for other purposes also might be synthesized.

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Chylomicron-like Particles in Severe Hypertriglyceridemia

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ABSTRACT

Plasma lipoproteins in a diabetic patient with severe hypertriglyceridemia and turbid, milky plasma were studied and compared with those found in three primary type V hyperlipoproteinemic and in three normal subjects after a fatty meal. All subjects had normal post-heparin lipolytic activity. Lipoprotein electrophoresis of the patient's plasma revealed no chylomicron band, and upon short-time ultracentrifugation the patient's supernatant showed most lipid staining in the pre-β-lipoprotein region. This supernatant, which in type V hyperlipoproteinemia as well as in normal subjects after a fatty meal, contains a large proportion of apolipoprotein B-48 (of intestinal origin), was found in our patient to contain mostly apolipoprotein B-100 (of liver origin). Upon incubation of normal washed platelets with chylomicrons derived either from type V hyperlipoproteinemic or from normal subjects after a fatty meal, platelet aggregation was decreased, whereas the chylomicron-like particles from the patient increased platelet activity. It is, thus, suggested that the triglyceride-rich, cholesterol-poor lipoproteins are of liver and not of intestinal origin.
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INTRODUCTION

Chylomicrons, the intestinally derived lipoproteins, usually are separated from plasma quantitatively by short-time ultracentrifugation and qualitatively by electrophoresis on cellulose acetate (1). The chylomicrons are the exogenous triglyceride-rich lipoproteins (TRP), and in comparison with the very-low-density lipoproteins (VLDL), which carry the endogenous triglycerides, they have a higher triglyceride/cholesterol ratio (2). They also contain apolipoprotein B-48, which is not found in VLDL (3), which contains only apolipoprotein B-100. Plasma chylomicrons can be separated from fasting type V hyperlipoproteinemic patients as well as from normal subjects after a fatty meal. In hyperchylomicronemia secondary to diabetes, overproduction of VLDL has been demonstrated (4), and the increased hepatic triglyceride synthesis may result in the production of VLDL–rich in triglycerides–which will fall into the chylomicron density. During high-carbohydrate feeding, the endogenous hypertriglyceridemia is due partly to chylomicron-like particles (5).

Platelets possess specific receptors for plasma lipoproteins (6), and upon incubation of plasma lipoproteins with isolated platelets, VLDL and low-density lipoprotein increase platelet aggregation and [^{14}C]serontonin release, whereas high-density lipoprotein decreases platelet function (7-10). Increased platelet function in hypercholesterolemia (11) and decreased platelet activity in type V hypertriglyceridemia recently have been demonstrated (12). Since chylomicrons and VLDL have an opposite effect on platelet function (8,12), we used the platelets in order to analyze the characteristics of TRP from various subjects.

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MATERIALS AND METHODS

Subjects

All subjects were males aged 35-50 years with normal body weight. Three normal subjects were given a fatty meal consisting of 175 g cream, 150 g cheese, 100 g olives, 100 g milk, 60 g bread and 20 g butter. This formula provided 41 g protein, 53 g carbohydrate, 77 g fat and 0.2 g cholesterol, with a caloric content of 1064 kcal and a polyunsaturated/saturated ratio of 0.086. Blood was taken 4 hr after the meal. Three patients with primary type V hyperlipoproteinemia were characterized by a significant hypertriglyceridemia associated with fasting chylomicronemia and increased plasma VLDL concentration. Patient S.B., 45 years old, suffered from diabetes, with fasting plasma glucose of 19 mmol/l, and severe hypertriglyceridemia. His liver, renal and thyroid functions were normal.

Venous blood was collected from all subjects after 14 hr of fasting into 1 mmol/l disodium EDTA. Blood also was drawn from five normal subjects after 14 hr of fasting into ACD (1.4% citric acid, 2.5% sodium citrate, and 2% dextrose) for platelet studies.

Lipoproteins

Triglyceride-rich lipoproteins were isolated by ultracentrifugation at 26,000 × *g* for 1 hr at 4 C. The TRP were washed with saline solution (d = 1.006 kg/l) under similar ultracentrifugation

conditions. The cholesterol and triglyceride of the plasma as well as the TRP were determined by enzymatic methods (13,14) in a centrifugal fast auto-analyzer (Gemsaec, Fairfield, New Jersey). Plasma and TRP electrophoresis were analyzed on cellulose acetate as described elsewhere (15). Post-heparin lipolytic activity was determined according to the method of Boberg and Carlson (16).

Sodium-dodecyl-sulfate/polyacrylamide Gel Electrophoresis (SDS-PAGE)

Three hundred microliters of TRP were delipidated twice with 10 ml ethanol/ether (3:1, v/v) for 24 hr at -20 C (17). The protein was solubilized in 350 μl of Tris buffer (pH 6.8) containing 5% SDS and 7% mercaptoethanol and was incubated overnight at 37 C. Twenty microliters of solubilized protein were analyzed by 3.5% SDS-PAGE by the method of Laemmli (18). Gels were fixed and stained in 0.25% Coomassie Blue G in methanol/acetic acid/water (45/10/45, v/v/v). At an optical density of 560 nm the gels were scanned using a Gilford spectrophotometer, and the areas under the protein peaks were determined by weighing.

Polyacrylamide Gel Isoelectric Focusing (19)

The TRP were delipidated using 4.2 mmol/l of 1,1,3,3-tetramethylurea, and 100 μg of protein were applied to each gel (8 × 0.6 cm). Each sample was treated with 50 mmol/l dithiotreitol, 0.5% Triton X-100 and 5% sucrose (final concentration). Gels (10%) were prepared with 6 mmol/l urea, 10% acrylamide and 0.2% bisacrylamide, using 3% ampholine (Bio-lyte 3.5/10, Bio-Rad, Richmond, California), pH 3.5-10, 0.06% ammonium persulfate and 0.006% tetramethylethylenediamine. The anode solution was 10 mmol/l phosphoric acid, and the cathode solution 0.2 mol/l sodium hydroxide. The samples (200 μl) were applied onto the gels through the electrolyte solution, and electrophoresis was performed at 250 V for 18 hr at 4 C. To determine the pH gradient, 2-mm segments of unfixed and unstained gels were transferred into a glass tube containing 2 ml of distilled water, and the pH of each segment was determined after 24 hr at room temperature. The gels were simultaneously fixed and stained for 2 hr at 45 C with 0.25% Coomassie Blue G in 50% methanol and 10% acetic acid. Destaining was performed using acetic acid/methanol/water (1:3:5, v/v/v).

Platelet Preparation

Four and one-half volumes of blood were added to 0.5 vol of ACD in plastic tubes. Samples were centrifuged at 200 × *g* for 10 min at 23 C. Platelet-rich plasma (PRP) was separated with plastic tips. Acetic acid (10 mmol/l) was added to a PRP solution, which was then centrifuged at 2,000 × *g* for 10 min. The supernatant was removed and the pellet resuspended in an equal volume of Hepes buffer (5 mmol/l Hepes, 137 mmol/l NaCl, 2.7 mmol/l KCl, 1.2 mmol/l $MgCl_2$, 12 mmol/l $NaHCO_3$, 0.4 mmol/l $NaH_2PO_4 \cdot H_2O$, and 0.6 mmol/l $C_6H_{12}O_6$, pH 7.4). The centrifugation and dilution with buffer were repeated and a washed-platelet preparation thus obtained.

Incubation Procedure

Washed platelets (200,000/μl) from normal, healthy subjects were incubated for 30 min at 37 C with similar concentration (0.15 and 0.30 mmol/l triglyceride) of TRP derived from normal subjects after a fatty meal, type V hyperlipoproteinemics, and patient S.B. The incubation medium contained Hepes buffer and 0.35% albumin. At the end of the incubation, collagen (1 μg/ml)-induced platelet aggregation was determined.

Platelet Aggregation

Platelet aggregation was studied in an aggregometer (Chrono-Log, Broomall, Pennsylvania) according to the method of Born (20). Collagen (1 μg/ml; Hormone Chemie, Munich, West Germany) was the aggregating agent. Platelet suspension (450 μl) was placed in a siliconized cuvette 8 mm in diameter and was stirred by means of a plastic bar. The cuvette was placed in the aggregometer, as was a cuvette containing the same amount of Hepes buffer as a control. Fifty microliters of the aggregating agent were added, and the light transmittance was monitored by a Heath Servo Recorder (Model EV-20B).

The Wilcoxon rank test was used for statistical analysis.

RESULTS

Post-heparin lipolytic activity was found to be in the normal range (6.9-9.0 μmol free fatty acids/ml/h) for all subjects. Table 1 demonstrates plasma and TRP lipid levels in normal subjects 4 hr after a fatty meal, in patients with primary phenotype V hyperlipoproteinemia, and in patient S.B. In comparison to the normal subjects, patient S.B. and the phenotype V hyperlipoproteinemic patients showed high levels of both triglycerides and cholesterol in plasma and in the TRP fraction (Table 1). Increased triglyceride/cholesterol ratio in both plasma and TRP was found in phenotype V hyperlipoproteinemia (Table 1), whereas in patient S.B. this ratio was

TABLE 1

Lipid Concentration in Plasma and in Triglyceride-rich Lipoproteins from Normals, Type V Hyperlipoproteinemics and Patient S.B.[a]

Group	No.	Plasma (mmol/l)			Triglyceride-rich lipoproteins (mmol/l)		
		TG[b]	Chol[b]	TG/Chol	TG	Chol	TG/Chol
Normal[c]	1	2.26	4.95	0.46	1.31	1.66	0.79
	2	2.82	4.27	0.66	1.36	1.27	1.07
	3	3.31	4.74	0.70	1.73	1.58	1.09
Type V	1	17.49	9.45	1.85	11.55	4.22	2.74
	2	14.14	8.05	1.76	8.49	3.24	2.62
	3	11.51	8.31	1.38	6.90	2.20	3.14
Patient S.B.		10.81	10.41	1.04	7.64	3.12	2.45

[a]All results represent mean of three determinations.

[b]TG=triglycerides; Chol=cholesterol.

[c]Normal subjects 4 hr after a fatty meal.

between normal and type V values.

Even though the plasma of patient S.B. was milky and a cream layer was found after leaving the plasma at 4 C overnight, on lipoprotein electrophoresis no lipid-staining band (corresponding to chylomicrons) could be found in the origin (Fig. 1A), but a heavily lipid-stained band was migrated faster than normal VLDL. Both in normal subjects after a fatty meal and in type V hyperlipoproteinemics, chylomicrons were stained at the origin (Fig. 1A). Analysis of the ultracentrifugally derived TRP (Fig. 1B) revealed that in normal subjects after a fatty meal it stained at the origin. In type V hyperlipoproteinemics a small fraction appeared, also

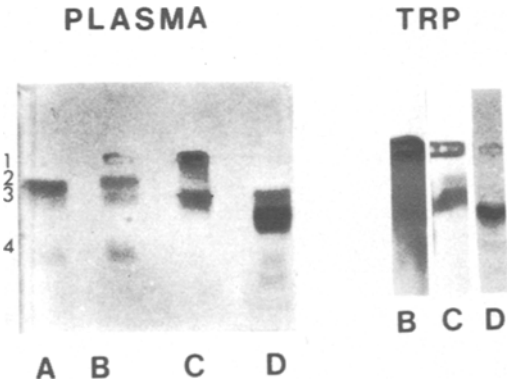

FIG. 1. Lipoprotein electrophoresis on cellulose acetate of plasma and of triglyceride-rich lipoproteins (TRP) derived from normal fasted subjects (A), normal subjects 4 hr after a fatty meal (B), type V hyperlipoproteinemics (C) and patient S.B. (D). 1, Origin (chylomicrons); 2, β-LP; 3, pre-β-LP; 4, α-LP; LP, lipoproteins; TRP, TRP obtained after ultracentrifugation of the plasma at 16,000 × g for 1 hr at 4 C.

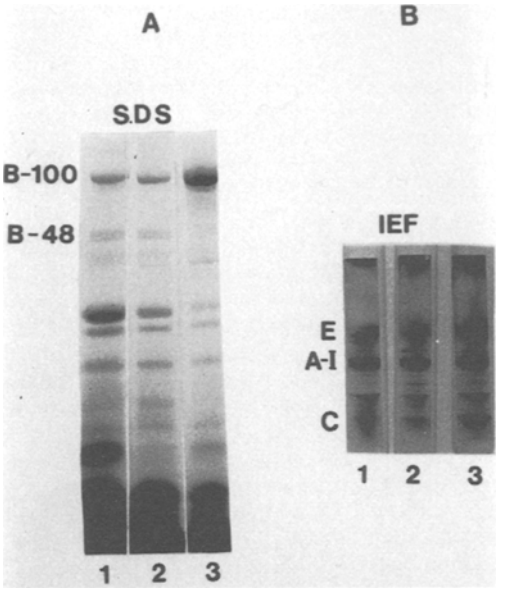

FIG. 2. Triglyceride-rich apolipoprotein electrophoresis on polyacrylamide gels using (A) sodium-dodecyl-sulfate (SDS) and (B) isoelectric-focusing (IEF) analysis. 1, Normal subjects after a fatty meal; 2, type V hyperlipoproteinemic subjects; 3, patient S.B.; C, A-I, and E, apolipoproteins C, A-I, and E, respectively.

in the VLDL region, but in patient S.B. most of the TRP migrated faster than normal VLDL. The TRP derived from all groups was delipidated and analyzed by PAGE (Fig. 2). Figure 2A demonstrates that, whereas TRP from normal subjects after a fatty meal and from type V hyperlipoproteinemic subjects contained apo B-100/apo B-48 in a proportion of 3.5:1 and 4.2:1, respectively, in patient S.B. a much greater

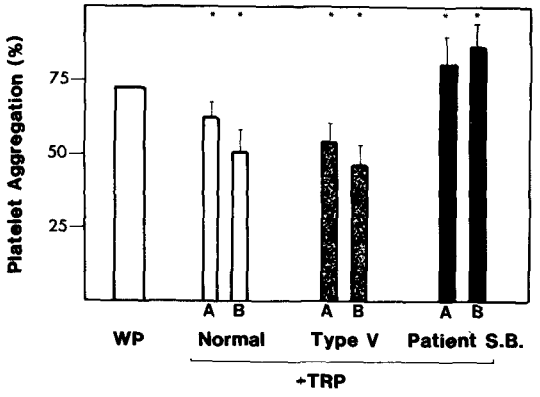

FIG. 3. Effect of triglyceride-rich lipoproteins (TRP) from normal subjects after a fatty meal (1), type V hyperlipoproteinemics (2), and patient S.B. (3) on normal washed-platelet (WP) aggregation. Normal-derived WP were incubated with TRP [0.15 (A) and 0.30 (B) mmol triglyceride/l] for 30 min at 37 C, and collagen (1 μg/ml)-induced platelet aggregation was then determined. $P < 0.01$ (versus WP).

proportion of apo B-100 was present (apo B-100/apo B-48 ratio of 15:1). Isoelectric-focusing analysis (Fig. 2B) revealed that TRP derived from patient S.B. contained increased concentration of apo C's in comparison to the other TRP studied. The effect of TRP derived from all the studied groups on normal washed-platelet aggregation (induced by 1 μg/ml of collagen) was then determined (Fig. 3). TRP (0.15 mmol/l triglycerides) derived from either normal subjects after a fatty meal or from patients with type V hyperlipoproteinemia caused decreased platelet aggregation after 30 min of incubation with normal washed platelets (reduction by 16% and 33%, respectively), and this effect was concentration-dependent (Fig. 3). TRP derived from patient S.B., however, demonstrated the opposite pattern (Fig. 3), showing increased platelet aggregation.

DISCUSSION

Chylomicrons are the lipoproteins identified by their physical properties ($S_f > 400$) and their physiological origin from dietary fat. Under pathological or abnormal dietary conditions, however, chylomicron-like particles appear in the circulation, but they contain endogenous triglycerides. The plasma TRP, chylomicrons and VLDL are somewhat similar in composition, but the endogenous triglyceride carrier (VLDL) contains more cholesterol and less triglyceride level (2). Our results show that the TRP derived from patient S.B. showed triglyceride/cholesterol ratio which fell between chylomicron and VLDL values (Table 1; Ref. 2). Patients with type V hyperlipoproteinemia have high plasma levels of both chylomicrons and VLDL, and they have a defect in triglyceride clearance (21) which may result from their abnormal TRP (Figs. 1 and 2). Our data demonstrated that the chylomicrons derived from type V patients demonstrate a significant increment in triglyceride/cholesterol ratio in comparison to those lipoproteins obtained from normal subjects after a fatty meal (Table 1). This phenomenon probably results from a sustained inhibition of hepatic cholesterogenesis by chylomicron remmants (21) in type V patients, whereas in normal subjects chylomicron levels increase only transiently after the fatty meal, with only minimal suppression of cholesterol synthesis. The increased TRP triglyceride content in type V patients may result from both impaired clearance as well as enhanced triglyceride synthesis resulting from increased local free fatty acids by virtue of hepatic lipase activity. This possibility is suggested since the type V TRP show not only intestinal characteristics, but also hepatic features. Figure 1B demonstrates the presence of some pre-β-particles in type V TRP plasma, and FIg. 2A demonstrates some higher levels of B-100/B-48 than those found in normal-derived chylomicrons (after a fatty meal). The TRP derived from patient S.B. was different from the other TRP, since it showed almost no chylomicron band, but a lipid-stained band migrating faster than normal VLDL (Fig. 1). It also contained a very small amount of apo B-48 (Fig. 2A), the intestinal apolipoprotein, and showed highly increased apo C concentration (Fig. 2B). This patient's TRP, although resembling the type V chylomicron–being triglyceride-rich, cholesterol-poor particles–probably are mostly of hepatic and not of intestinal origin (23).

Since plasma lipoproteins affect platelet function (7-11,24,25), we analyzed the in vitro effect of TRP on normal platelet aggregation (Fig. 3). We have demonstrated previously that chylomicrons inhibit platelet aggregation (12), whereas VLDL increases platelet activity (7-10). Figure 3 demonstrates that postprandial normal chylomicrons or type V TRP, when incubated with normal washed platelets, decrease platelet aggregation, but TRP derived from patient S.B. increased platelet aggregation, resembling the effect of VLDL (and not of chylomicrons) on platelet function. Thus, we suggest that this patient's TRP are abnormal VLDL. The effect of this patient's TRP on normal platelet function may result from their high apo B-100/apo B-48 ratio, since this is a characteristic of VLDL particle as opposed to chylomicrons. Our study suggests that the appearance of milky plasma

and the obtaining of "chylomicrons" by normal technical procedures may not necessarily mean the presence of the dietary fat-carrier intestinal lipoproteins, and this should be taken into consideration when treatment is considered.

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Free Fatty Acid Content of Human Milk: Physiologic Significance and Artifactual Determinants

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ABSTRACT

Analysis of human milk was conducted to determine if free fatty acids occur naturally or as a consequence of artifactual lipolysis after milk expression. Five mothers provided triplicate early morning milk samples on day 43 of lactation. Following extraction, lipid classes were separated by preparative thin layer chromatography and quantified by capillary gas liquid chromatography. Fresh milk samples collected with 20 volumes chloroform-methanol (1:1, v/v) were analogous in total free fatty acid level and profile of fatty acids to a duplicate sample collected with 0.4M EDTA and immediately frozen at −10 C. Low milk levels of free fatty acids appear to exist naturally. During days 4-37 of lactation, four serial milk samples from 15 mothers were collected and frozen with 0.4M EDTA. The concentration of free fatty acids in colostrum (0.03-0.5%, w/w) was lower than for subsequent days (0.3-2.5%, w/w). Additional samples were collected with and without a lipase inhibitor (0.4M EDTA) and subjected to routine collection and storage procedures. Significantly different fatty acid profile and higher levels of free fatty acids in milk collected without a lipase inhibitor added indicate that domestic freezing and/or thawing ruptures the fat globule membrane, allowing sn-1-stereospecific serum stimulated lipoprotein lipase contact with its triglyceride substrate. Standard procedures for collection of human milk for gavage fed infants appear to stimulate artifactual lipolysis of milk triglyceride and subsequent release of free fatty acids. The proposed relationship between dietary free fatty acids and prolonged, unconjugated hyperbilirubinemia in the newborn is discussed with regard to the significance of preintestinal lipolysis.
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INTRODUCTION

A recent review by Gaull et al. (1) points to a lack of definitive data regarding the quantitation of human milk lipid classes and constituent fatty acids. Although free fatty acids (FFA) are present in human milk (2), their origin has become controversial (3,4). The potential influence of collection and storage on increasing the level of FFA has been discussed recently (5). Bitman and coworkers (4) have indicated clinical concern regarding the feeding of domestically frozen human milk to newborn infants at risk for unconjugated hyperbilirubinemia. Wardell and colleagues (1984) also recently have questioned the appropriateness of feeding frozen milk to high risk low birthweight infants (6) following their findings that freezing milk significantly lowers triglyceride linoleic and linolenic acid content (7).

The present report is part of a study examining factors influencing lipid composition of early human milk (8,9). The design enabled examination of whether or not FFA occurs naturally in human milk and if the levels and profile of FFA present in milk can be related to transitions in human milk lipid content and/or to routine clinical and laboratory processing methods for milk collected.

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MATERIALS AND METHODS

Milk Collection and Lipid Analysis

Five mothers provided triplicate milk samples on day 43 postpartum. Milk specimens were obtained with either a Kaneson mechanical breast pump (Marshall Electronics Inc., Skokie, Illinois) or an Egnell electric breast pump (Egnell Inc., Cary, Illinois). Samples were collected with either 20 volumes of chloroform-methanol (1:1, v/v) or into 0.7 ml of 0.4M EDTA, or without a lipase inhibitor and frozen at −10 C (Table 1). A modified Folch extraction procedure was used. Each 3.7 ml milk sample was brought to 37 C quickly and extracted with chloroform-methanol (2:1, v/v) containing n-heptadecanoic acid. Half of this sample was reserved for total fatty acid analysis. Neutral lipids were separated on 1000μ silica gel G thin layer chromatography plates using petroleum ether/diethyl ether/formic acid (60:40:1.2, v/v/v) as the solvent system and double development for 60 min and 40 min, which enabled clear separation of lipid classes (cholesterol ester, triglycerides, free fatty acids, 1,3 di-

TABLE 1

Effect of Collection Procedures on the Free Fatty Acid Content of Human Milk[a]

Fatty acid (x̄% w/w)	Milk total fatty acids	Collection procedure[b]		
		Immediate extraction in chloroform	Frozen[c] with EDTA	Frozen without EDTA**
$C_{10:0}$	0.9	tr	tr	2.5
$C_{12:0}$	3.2	5.5	5.7	9.7
$C_{14:0}$	4.7	6.7	6.7	4.1
$C_{16:0}$	20.0	25.3	24.0	6.9
$C_{18:0}$	6.1	7.0	7.5	3.2
$C_{18:1\omega-9}$	35.7	31.6	31.5	42.4
$C_{18:2\omega-6}$	18.0	13.8	12.1	18.3
Total free fatty acids (% w/w) of total fatty acids	–	1.2 ± 0.06	1.3 ± 0.08	7.9 ± 0.16

[a]n = 5.

[b]Samples were collected from the same donors for each procedure.

[c]Initial storage temperature was −10 C.

** = $p < 0.01$ for milk collected with chloroform or with EDTA versus without EDTA.

glycerides, 1,2 diglycerides, cholesterol, monoglycerides and phospholipids). Separated lipids were visualized with 2.7 dichlorofluorescein under UV light. The phospholipid band was removed for phospholipid class analysis (Clandinin, M.T., and Chappell, J.C., unpublished data). Monoglyceride, diglycerides, triglyceride and cholesterol ester bands were spotted with n-heptadecanoic acid. FFA, monoglyceride, diglyceride and triglyceride bands and the duplicate total lipid sample were transmethylated with 10% (w/v) methanolic-HCl. Fatty acids were analyzed quantitatively by capillary gas-liquid chromatography. These analyses used 30-meter grade AA glass capillary columns coated with SP1000 (Supelco Inc., Bellefonte, Pennsylvania) and provided data for positional isomers for over 50 fatty acids from eight to 24 carbons in chain length (Chappell, J.E., Clandinin, M.T., and Kearney-Volpe, C., unpublished data).

Effect of Gestational Age and Lactational Stage

Milk samples were obtained from eight mothers who delivered infants between 27-33 wk of gestation (PT) and seven women who delivered at term (FT). Each mother provided four early morning samples during the first six weeks of lactation (Fig. 1); on days 16 and 37, postpartum samples of milk were aliquoted and combined to provide a pooled milk sample reflective of the 24-hr period (Table 2). Milk volume lactated and time interval since the last expression or nursing were recorded. Aliquots (10 ml) of milk were immediately removed by the mothers, mixed with 0.7 ml of 0.4M EDTA and frozen at −10 C. Samples were transported on dry ice to the laboratory and stored at −80 C for analysis. Procedures for milk extraction and analysis are described above.

Influence of Milk Processing on Free Fatty Acid Content

Complete early morning expressions of milk were collected from five women on days 43 and 50 postpartum. Immediately after the volume was recorded, the 10 ml sample was mixed with 0.7 ml of 0.5 M EDTA. The remaining milk was not mixed with EDTA. Sample pairs with or without EDTA were stored at −10 C or −80 C, warmed to 22 C or 37 C, followed by one or two additional freeze/thaw procedures (Table 3). Procedures for extraction and lipid analysis were as described above.

Statistics

Procedures for analysis of variance, covariance, correlation and paired t-test comparisons (10) were used to determine the effect of gestational age, lactational stage, milk collection and processing procedures on milk FFA content.

RESULTS AND DISCUSSION

The present study was designed to differentiate between effects endogenous to the mammary gland and subsequent collection proce-

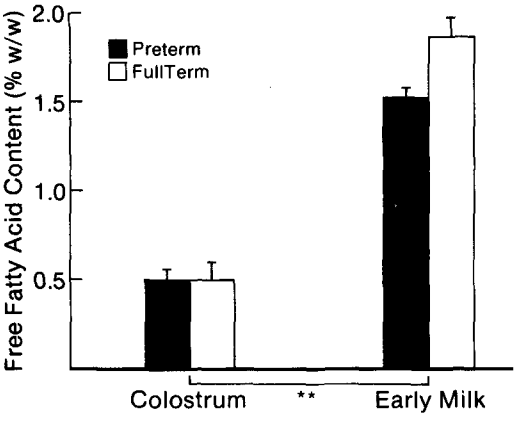

FIG. 1. Free fatty acid levels in milk as a function of gestational age and lactational stage. Values illustrated represent the mean ± SEM. ** = $p < 0.01$.

TABLE 2

Free Fatty Acid Levels in Early Human Milk[a]

	Full term	Preterm
Total fatty acids[b] (gm/dl ± SEM)	2.4 ± 0.10	3.3 ± 0.14*
Free fatty acids[c] (mg/dl ± SEM)	46.5 ± 0.08	49.3 ± 0.07
% w/w ± SEM	1.9 ± 0.07	1.5 ± 0.04

[a]n = 15.

[b]Total fatty acids and free fatty acids were determined for a representative 24 hr milk sample.

[c]Free fatty acids were analyzed in 16 pooled PT and 14 pooled FT milk samples. Pooling of EDTA samples was done at the point of extraction. The number of within day expressions was 6.

* = $p < 0.05$.

TABLE 3

Influence of Processing Subsequent to Milk Collection on the Fatty Acid Composition of Free and Total Fatty Acids Present in Human Milk[a]

	Conditions of milk processing[b]		
Fatty acid ($\bar{x}$% w/w)	Free fatty acids** without EDTA frozen −10 C	Free fatty acids with EDTA (−10 −80 C) without EDTA (−80 C)[c]	Total fatty acids (−10 and −80 C)
$C_{10:0}$	3.1	0.6	1.5
$C_{12:0}$	10.1	6.1	5.6
$C_{14:0}$	4.7	8.9	7.5
$C_{16:0}$	7.7	25.6	22.3
$C_{18:0}$	4.5	9.0	6.1
$C_{18:1\omega-9}$	42.0	34.5	36.7
$C_{18:2\omega-6}$	11.9	6.2	9.0
Total free fatty acids ($\bar{x}$ ± SEM % w/w) of total fatty acids	7.7 ± 0.15-12.6 ± 0.28[d]	1.6 ± 0.05	(2.2 ± .2) gm/dl ± SEM

[a]n = 5.

[b]Samples were collected from the same donors for each procedure.

[c]Collection with EDTA or without EDTA not significantly different if frozen immediately at −80 C.

[d]Following the second freeze/thawing total free fatty acid level was increased. A third freeze/thaw produced no subsequent alteration of profile or level of FFA.

** = $p < 0.01$ for milk collected without EDTA versus with EDTA.

dures on the profile and levels of FFA in human milk.

The fatty acid pattern and content of milk FFA were similar for paired samples collected immediately in chloroform-methanol or with EDTA (Table 1). The proportions and levels of FFA in milk collected without a lipase inhibitor were significantly different ($p < 0.01$) when compared to analogous samples collected with a lipase inhibitor (Table 1). The distribution of fatty acids in human milk triglyceride is asymmetric (11) with a significant proportion of $C_{12:0}$, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ located in the sn-3, sn-2, sn-1, and sn-1 positions, respectively (12,13). The relative decrease of $C_{16:0}$ and elevation of $C_{18:1}$ content of these samples collected without EDTA suggest activation of sn-1-serum stimulated lipoprotein lipase (14,15). An inverse relationship for $C_{16:0}$ and $C_{18:1}$ was observed when diglyceride and monoglyceride fractions of milk samples collected without EDTA were analyzed. The fatty acid profile

and levels of monoglyceride and diglycerides in non-EDTA treated milk samples differed significantly from EDTA samples.

Medium chain saturated and long chain monounsaturated species are hydrolyzed by lipoprotein lipase in preference to long chain saturated and polyunsaturated species (15), thus explaining the significant elevation of $C_{12:0}$ and decrease in $C_{18:0}$ as FFA, relative to the total fatty acid profile (Table 1). Recently, it has been shown that freezing of human milk results in loss of bile salt stimulated esterase dependence on bile salts for activation (16). Therefore, the authors have suggested that given the high ratio of bile salt stimulated esterase activity relative to serum stimulated lipoprotein lipase activity, the bile salt stimulated esterase may be responsible for lipolysis of milk triglycerides that is associated with storage of milk (16). However, if bile salt stimulated esterase and bile salt stimulated lipase (17) are functionally similar enzymes (16), the low positional specificity of the enzyme would not release the profile of fatty acids observed in the present study (Table 1).

Milk collections were made and frozen at −10 C for up to six weeks, as these conditions (i.e. sampling by the mothers, home refrigerator-freezer temperatures and realistic times for collection of samples) were found to be most feasible given the longitudinal aspect of the overall study. Comparisons between sampling procedures supported initial calculations of the molar concentration of EDTA required to chelate the maximum reported milk calcium content and thereby prevent calcium activation of lipoprotein lipase. Storage with 1 M NaCl or sodium taurocholate (14), or heating to 55 C (4,5) as methods of inhibition were not found to be appropriate for subsequent analysis.

The 2.8% FFA value reported by Czegledi-Janko (1965) has been dismissed by Gaull et al. due to limitations in methodology (1). Subsequent milk levels of FFA reported range from 0.08% w/w (4) to 0.4% w/w (2). These levels of FFA are markedly lower and different in profile (3) from those observed in the present study. To ensure quantitation in the present study, n-heptadecanoic acid was added during the initial extraction procedure. If lipolysis of triglyceride is ongoing as the total expression (averaging 85 ml) is collected, then it may be argued that the resultant FFA level reflects endogenous levels present in suckled human milk. Low concentrations of cholate and chenodeoxycholate have been reported in colostrum (0.17-0.75 μmol/l) and later milk (0.25-4.38 μmol/l) (18). The range of FFA reported for human milk and the classification of "stable" and "unstable" milks following freezing (16) should perhaps be re-examined in view of the milk content of cholate and chenodeoxycholate and subsequent induced activation of bile salt stimulated lipase/bile salt stimulated esterase (19). The level and profile of FFA may reflect the partitioning of triglyceride biosynthesis and hydrolysis within the mammary gland.

Effect of Gestational Age and Lactational Stage on Milk FFA Content

The FFA content of PT and FT milk was similar (Fig. 1), following a corresponding increase with the transition from colostrum (0.50 ± 0.01% w/w) to early milk collected on day 7 (1.7 ± 0.06% w/w) ($p < 0.01$). From day 11 to 37 there were no significant differences in milk FFA content. This trend parallels the pattern of increase reported in total milk fatty acid levels associated with establishment of lactation (2,9,20,21). The increase in FFA levels in early lactation is the inverse of the pattern predicted if bile salt stimulated esterase (16) was active in the release of fatty acids. Analysis of covariance indicated that milk volume was not a significant determinant of milk FFA content for each lactational stage and gestational age examined. Free fatty acid levels lactated on days 16 and 37 postpartum and representative of the 24 hr period (1.6 ± 0.05% w/w) were similar in FFA content to single early morning expressions (Fig. 1; Table 2).

Differences in macronutrient composition between PT and FT milk (8,22-24), albeit controversial (25,26), have been interpreted by some (23,27,28) to indicate functional immaturity of the PT mammary gland. If milk FFA levels reflect the capacity of the mammary gland for lipid synthesis, then the lack of difference between PT and FT milk FFA content (Table 2) does not appear to support this hypothesis. Indeed, a negative correlation ($r = -0.675$; $p < 0.05$) was found between PT milk total fatty acid content and FFA levels. This observation was not apparent for FT milk. However, a negative correlation was observed between the time period elapsed since the last milk expression ($r = -0.6$, $p < 0.05$) and levels of FFA in FT milk. These observations perhaps indicate a difference in rates of PT and FT mammary gland lipogenesis.

Serum stimulated lipoprotein lipase levels for PT and FT colostrum have been reported to be similar and increase significantly with established lactation (16). The increase in enzyme activity was greater in milk of term mothers than in PT milk for the first three weeks postpartum. If milk serum stimulated lipoprotein lipase levels reflect mammary gland

clearance of chylomicrons, then the pattern of milk FFA levels (Fig. 1) may partially reflect removal of fatty acids from plasma for milk lipid synthesis. In this regard, colostrum FFA levels of $C_{18:1(\omega-9)}$ were significantly higher for both PT and FT milks when compared with subsequent days studied ($p < 0.01$).

Influence of Collection Methods on Milk FFA Content

When milk samples were subjected to freezing temperatures of −10 C or −80 C and 1,2 or 3 freeze/thaw procedures it was found that when samples were warmed to 22 or 37 C prior to aliquoting for extraction no significant differences in FFA levels occurred. Consistency between duplicate analyses was, however, markedly improved at 37 C. Freezing at −80 C inhibited serum stimulated lipoprotein lipase activity as no significant difference occurred between paired samples collected with or without EDTA and immediately stored at −80 C (Table 3). The second thawing after storage at −10 C without EDTA increased total milk FFA levels ($p < 0.05$), although no marked change in fatty acid composition was observed for the major fatty acids. Subsequent freezing and thawing produced no further alteration of profile or milk level of FFA (Table 3). As neither freezer temperature, freeze/thawing nor warming temperatures produced any effect on either the total fatty acid content of milk or the profile of total fatty acids, it may be assumed that previously reported changes in milk content of $C_{18:2(\omega-6)}$ and $C_{18:3(\omega-3)}$ (7) following freezing represent shifts in fatty acid composition of lipid classes as a result of activated lipases and not decreased "essential fatty acid availability" in frozen milk (7).

It must be noted, however, that 7 of the 105 EDTA samples collected in this present report had FFA levels of greater than 4% (w/w) and were therefore considered 'damaged.' We have observed that when the level of FFA is 3.5-4.0 or greater, the profile of these FFA indicate significant serum stimulated lipoprotein lipase activity. The results were observed primarily on day 16 postpartum and in FT milk. These levels of milk FFA may be partially explained by the finding that lipoprotein lipase appears to have significant, maximal activity during the third week of lactation in FT milk (16). Examination of rates of lipid synthesis and influence of maternal diet on serum stimulated lipoprotein lipase activity and Apo CII in human milk may serve to explain these variations in milk lipids further.

Many preterm infants by virtue of immaturity are not fed at the breast. In the Neonatal Intensive Care Unit at the Hospital for Sick Children we have noted that standard handling procedures for milk (n = 11) may mean that up to 20% of the total fat fed is in the FFA form. These levels of milk FFA occur as a consequence of collection procedures rather than as a phenomenon of lactation and may be one of the factors responsible for the efficiency of lipid absorption observed for gavage fed high risk infants when fed non-heat treated, previously frozen mothers' milk. Given the nonsterospecific action of bile salt stimulated lipase in human milk fat digestion (29), the physiologic significance of palmitic acid in the sn-2 position of the milk triglyceride is at present unclear. However, as a result of the specificity of serum stimulated lipoprotein lipase, palmitic acid present in these milks would be predominantly within the mono or diglycerides formed.

Concern has been raised regarding feeding significant levels of free fatty acids (4), given the inverse relationship between plasma nonesterified fatty acids and UDP-glucuronyl transferase activity in the newborn (30). A hypothetical mechanism has been proposed for the relationship between increased breast milk FFA and prolonged unconjugated hyperbilirubinemia. Extensive lipolysis prior to duodenal absorption results in a relative deficiency of glycerol and subsequently diminished resynthesis of triglyceride. FFA are then absorbed into the portal system, enter the liver cell and inhibit UDP-glucuronyl transferase synthesis.

Approximately 30-40% (w/w) of formula and human milk lipid is reported to be hydrolyzed by lingual lipase in the stomach of the newborn (31,32). This activity is believed to be compensatory to the suboptimal intestinal digestive capacity of the newborn. Intragastric hydrolysis produces mainly partial glycerides and FFA, amphiphilic substances which assist in emulsification of fat in the stomach and intestine (31). Therefore, it would appear that preintestinal lipolysis actually facilitates efficient absorption of lipid in most newborns without the sequelae of prolonged unconjugated hyperbilirubinemia. The clinical syndrome of breast-milk jaundice has been recognized by many (33,34-36). However, its etiology remains controversial (33,35-38). Elevated levels of FFA found by Poland and coworkers in breast milk of jaundiced infants has not been noted by others (36,38). The significant influence of milk storage (5) and lactational stage on milk levels of FFA and lipoprotein lipase activity (16,39) may partially explain these conflicting findings.

In the present study, the level and composi-

tion of FFA was determined on repeated milk samples obtained from the same individuals. Controlled sampling procedures allowed differentiation between endogenous and artifactual FFA levels and fatty acid profiles. Examination of the profile and levels of milk FFA may serve to identify the partitioning of the fatty acid pool derived from endogenous and exogenous sources for milk lipid synthesis. Increased milk FFA levels following 'routine' storage procedures may facilitate lipid absorption in the gavage fed newborn.

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Metabolism and Cholestatic Effect of 3α-Hydroxy-7ξ-Methyl-5β-Cholanoic Acid

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ABSTRACT

3α-Hydroxy-7ξ-methyl-5β-cholanoic acid (7ξ-methyl-LA) was infused intravenously into bile fistula hamsters to investigate its metabolism and effect on the bile flow as compared with lithocholic acid. Following infusion of the labeled bile acids, bile was collected quantitatively to allow measurement of bile flow and bile acid composition. More than 80% of radioactivity was recovered in bile within 4 hr. 7ξ-Methyl-LA and lithocholic acid in bile were present as the taurine and glycine conjugates; no free bile acids were detected. 7ξ-Methyl-LA was neither hydroxylated nor metabolized to any measurable extent, though lithocholic acid was 7α-hydroxylated to chenodeoxycholic acid (30-45%).

At the infusion rate at which lithocholic acid induced a severe cholestasis (264 nmol/min), 7ξ-methyl-LA did not decrease the bile flow. In fact, the infusion of 7ξ-methyl-LA produced a mild choleresis under conditions where endogenous bile acid excretion was not changed appreciably compared to control infusions with albumin.

It is concluded that 7ξ-methyl-LA is not metabolized in the hamster but is conjugated with taurine and glycine, and that the introduction of a methyl group at the 7-position of lithocholic acid appears to alleviate the cholestatic effect of lithocholic acid in the hamster.
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INTRODUCTION

It is known that some monohydroxy bile acids, such as lithocholic acid, induce cholestasis in experimental animals (1-4). Cholestasis can occur even when the bile acids are conjugated with taurine or glycine, which presumably increases their water solubility. Most of the compounds which are known to induce cholestasis have a hydroxyl group at the 3-position of the steroidal A ring and are structurally similar to cholesterol. Possible mechanisms of lithocholic acid-induced cholestasis have been suggested not only on the basis of its physical properties but also by considering its hemolytic and inflammatory properties (5-9).

Recently, we have reported the synthesis (10) and metabolism (Une et al., Biochim. Biophys. Acta, in press) of two bile acid analogs, namely, 7β-methyl-chenodeoxycholic (7β-methyl-CDA) and 7α-methyl-ursodeoxycholic acid (7α-methyl-UDA) in hamsters. We found that these dihydroxy bile acids were largely excreted unchanged into feces, but some metabolism to a more polar product (probably trihydroxy bile acids) and a less polar product, 7ξ-methyl-LA, was also observed. In contrast, chenodeoxycholic acid was almost completely dehydroxylated by the intestinal bacteria flora to give lithocholic acid.

In this paper, we compare the metabolism and cholestatic properties of lithocholic acid and 7ξ-methyl-LA.

MATERIALS AND METHODS

Animals

Male golden Syrian hamsters (Engle Labs, Farmersburg, Indiana), weighing between 100 and 140 g, were maintained for at least two weeks on rodent chow. The animals were given food and water ad libitum. All animals were operated on between 8 and 9 a.m. Each hamster was anesthetized with sodium pentobarbital and ethyl ether, followed by bile duct cannulation with a polyethylene cannula (PE 10, inside diameter 0.28 mm). An infusion cannula (PE 10) subsequently was introduced into the femoral vein. Since the hamster has a gallbladder, cholecystectomy was necessary to obtain complete collections of bile.

Infusion Experiments

Metabolism of 7ξ-methyl-LA. For intravenous infusion the bile acids were prepared as follows: labeled lithocholic acid or 7ξ-methyl-LA (500-800 μg) was dissolved in methanol (40 μl), and this solution was rapidly injected into saline containing 10% bovine serum albumin (2.2 ml) and sonicated for 5 min.

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Saline was infused into the femoral vein for 1 hr prior to the administration of labeled compounds. Each compound was then infused for 2 hr at the rate of 250-400 μg/1.1 ml/hr. This was followed by saline which was infused for an additional 2 hr. Bile samples were collected every 30 min for 5 hr. The radioactivity of aliquots of each sample was determined by liquid scintillation counting (Beckman LS 8000). Another aliquot was analyzed by thin layer chromatography (TLC) using solvent system A, chloroform/methanol/acetic acid/water (13:4:2:1, v/v/v/v), before hydrolysis, and solvent system B, chloroform/ethyl acetate/acetic acid (9:9:1, v/v/v), after hydrolysis. A third aliquot was analyzed by gas liquid chromatography (GLC) on a SE-30 column (T_c = 250 C) and a HiEFF 8BP column (T_c = 240 C) as methyl ester trimethylsilyl ether derivatives after alkaline hydrolysis to measure bile acid excretion (Table 1). The relative retention times were: a) 1% HiEFF 8 BP; cholic acid, 1.00; lithocholic acid, 2.17; 7ξ-methyl-LA, 2.50, and b) 3% SE-30; cholic acid, 1.00; lithocholic acid, 0.77; 7ξ-methyl-LA, 0.90.

Cholestatic effect of 7ξ-methyl-LA. Unlabeled bile acids were prepared at a concentration of 5.28 μmol/0.7 ml as described above. The experiments were identical to those described above, except that infusions were carried out at the rate of 264 nmol/min for 20 min and bile was collected every 20 min. When different bile acids were infused into the same animal, saline was infused for 1 hr during the recovery phase before the next infusion of bile acid. Bile was collected quantitatively into calibrated tubes, and the bile acids were analyzed as described above.

Labeled Compounds

[7ξ-^{14}C]7ξ-Methyl-LA (sp. act. 4.53 × 10^5 cpm/mg), which is a mixture of the 7α- and 7β-methyl epimers (ratio about 1:1, as determined by GLC), was prepared as previously reported from this laboratory (10). [24-^{14}C] Lithocholic acid (sp. act. 59 mCi/mol) was purchased from New England Nuclear, Boston, Massachusetts. The purities of labeled compounds were better than 99% as determined by TLC with solvent system B.

RESULTS

[7ξ-^{14}C]7ξ-Methyl-LA or [24-^{14}C]lithocholic acid were infused intravenously into bile fistula hamsters in order to compare their

TABLE 1

Effect of Lithocholic Acid and 7ξ-Methyl-LA Infusion on Bile Flow and Bile Acid Excretion[a]

	Experiment number	Bile acid excretion rate (nmol/min)		Bile flow (μl/min)		
		Before[b]	After[c,d]	Before[b] B	After[c] A	A/B × 100 %
Control	1	33	26	6.9	6.5	94
	2	22	12	6.4	5.0	78
Lithocholic acid	3	31	9.9	5.5	3.5	64
	4	70	–[e]	4.0	0	0
	5	58	23	6.5	4.5	69
	6	39	–[e]	5.5	0	0
7-Methyl-LA	7	83	44	2.5	2.0	80
	8	78	82	6.0	7.0	117
	9	38	45	3.5	4.0	114
	10	22	46	5.5	7.0	127
	11	57	90	6.5	8.3	128

[a]Infusion rate of bile acids was at 264 nmol/min for 20 min. In the control group, albumin was infused for 20 min.

[b]Mean bile flow or bile acid excretion (by GLC) for 20 min before infusion of bile acids or albumin.

[c]Mean bile flow or bile acid excretion (by GLC) for 20 min after infusion of bile acids or albumin.

[d]Lithocholic acid and 7ξ-methyl-LA comprised 9-29% and 33-75% of total bile acids, respectively.

[e]Too low to analyze.

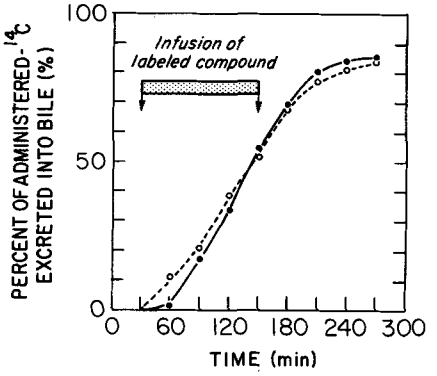

FIG. 1. Biliary excretion of radioactivity in bile fistula hamsters after intravenous infusion of labeled lithocholic acid (LA) and 7ξ-methyl-LA. —•— 7ξ-methyl-LA, ---o--- LA, Each value represents the mean of data from two male hamsters. Infusion period: 1) saline 0-30 min; 2) labeled bile acid 30-150 min; 3) saline 150-270 min.

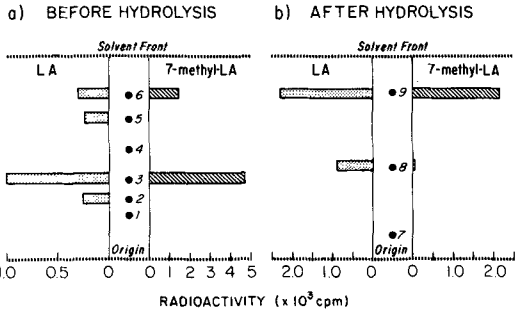

FIG. 2. Radio-TLC analysis of bile recovered after intravenous infusion of a) [24-^{14}C]lithocholic acid (LA) or b) [7ξ-^{14}C] 7ξ-methyl-LA. The radioactivity in the various bile acid components of the lithocholic acid infused animals is shown in the left panels (before hydrolysis as the conjugates and after hydrolysis as the free acids). The radioactivity in the various bile acid components of the 7ξ-methyl-lithocholic acid animals is shown in the right panels (before hydrolysis as the conjugates and after hydrolysis as the free acids). The following solvent systems were used: before hydrolysis, solvent system A; after hydrolysis, solvent system B (see text). Reference compounds: 1) sodium taurocholate; 2) sodium taurochenodeoxycholate; 3) sodium taurolithocholate or sodium tauro-7ξ-methyl-lithocholate; 4) sodium glycocholate; 5) sodium glycochenodeoxycholate; 6) sodium glycolithocholate or sodium glyco-7ξ-methyl-lithocholate; 7) cholic acid; 8) chenodeoxycholic acid, and 9) lithocholic acid or 7ξ-methyl-lithocholic acid.

metabolism. The dose of bile acid used was 250-400 μg/hr, which is considerably less than the dose of lithocholic acid required to produce cholestasis (ca. 6300 μg/hr, see below). Bile was collected and analyzed for total radioactivity and radioactivity in biliary components using TLC. Recovery of the administered labeled compounds in the metabolism studies is shown in Figure 1. Each bile acid was rapidly extracted by the liver and excreted into bile within 30 min after injection. Under the conditions employed, the total recovery of 7ξ-methyl-LA and lithocholic acid in bile was better than 85%. Figure 2 shows the radio-TLC analysis of the bile acids in bile obtained during the infusion experiments. In the infusion experiments with either lithocholic acid or 7ξ-methyl-LA, radioactivity was present in fractions containing taurine and glycine conjugates, but not in the free bile acid fractions (Fig. 2a). There were no significant differences in taurine and glycine conjugation between 7ξ-methyl-LA and lithocholic acid (Fig. 2a). Radio-TLC of the hydrolysates of the bile following lithocholic acid infusion (Fig. 2b) revealed that lithocholic acid had been 7α-hydroxylated to chenodeoxycholic acid. In contrast, radio-TLC of the hydrolysates of the bile following 7ξ-methyl-LA infusion showed little hydroxylation products in the bile. With respect to 7ξ-methyl-LA, TLC results show only radiolabeled compounds (i.e., compounds containing a labeled 7-methyl group). Total recovery of these labeled compounds was greater than 85%. Therefore, if 7-demethylation had taken place it would account for less than 15% of administered 7ξ-methyl-LA.

In order to investigate the effect of lithocholic acid and 7ξ-methyl-LA on bile flow and bile acid excretion, infusion experiments were carried out using unlabeled compounds. Since cholestasis was observed consistently when lithocholic acid was infused at the rate of 264 nmol/min for 20 min, this infusion rate was used in the following studies: Table 1 summarizes the results obtained in 9 infusions of 7ξ-methyl-LA and lithocholic acid in 6 hamsters. In the control group, bile flow and bile acid output decreased only slightly with time. During the infusion of lithocholic acid (Figs. 3a and b), bile flow remained unchanged but fell precipitously during the next 20-min or 40-min period (Figs. 3a and b). Lithocholic acid caused a significant decrease in bile acid excretion due to its inhibition of bile flow. The duration of the cholestatic effect induced by lithocholic acid was relatively great, and return to normal bile flow was not observed during the recovery phase of the experiments (Fig. 3a). On the other hand, 7ξ-methyl-LA infused at the same rate (264 nmol/min) as lithocholic acid caused no cholestasis and even tended to stimulate bile flow. 7ξ-Methyl-LA comprised about 33-75% of total bile acids excreted into bile after infusion,

and this was reflected in an increased total bile acid excretion. The choleretic effect of 7ξ-methyl-LA was minimal in comparison with that of an equal amount of sodium taurocholate, which produced a significant increase in bile flow (Fig. 3b).

DISCUSSION

Infusion of either [7ξ-^{14}C] 7ξ-methyl-LA or [24-^{14}C] lithocholic acid resulted in recovery of more than 85% of the administered radioactivity in the bile within 4 hr. This suggests that these bile acids were extracted effectively by the liver and rapidly excreted into the bile. TLC analysis of the bile samples revealed that both lithocholic acid and 7ξ-methyl-LA were conjugated completely with taurine and glycine. Neither free nor sulfated bile acids were detectable in the bile following lithocholic acid or 7ξ-methyl-LA infusion. Demethylation of [7ξ-^{14}C] 7ξ-methyl-LA would result in no radioactivity in the bile acid fractions. An important difference was observed by further analysis of the bile after hydrolysis. Lithocholic acid was found to be 7α-hydroxylated to give chenodeoxycholic acid in amounts ranging from 30% to 45%. This is comparable with the results previously reported by Emerman and Javitt (11) and King and Schoenfield (2). In contrast, 7ξ-methyl-LA was not rehydroxylated to a more polar compound. The presence of a methyl group at the 7-position apparently prevented the 7α-hydroxylation of this bile acid. Though 7ξ-methyl-LA used in this study was a mixture (about 1:1) of 7α- and 7β-methyl isomers, it seems unlikely that 7α-rehydroxylation occurs with only one of the isomers because the isomeric ratio remained unchanged before and after infusion. It appears that absence of 7α-rehydroxylation is not due to the epimeric configuration but rather to the presence of a methyl group at the 7-position.

It is widely known that lithocholic acid decreases bile flow in the hamster (2,9) and other experimental animals (9,12), even though it can be metabolized to more polar compounds, such as chenodeoxycholic acid and other polyhydroxylated bile acids. Other monohydroxy bile acids also have been shown to exert cholestatic effects (13). As shown in Table 1, a cholestatic effect of lithocholic acid was evident when it was infused at the rate of 264 nmol/min for 20 min. The cholestatic effect was not observed immediately during the infusion of lithocholic acid, but diminution of bile flow usually was observed during the next 20-min or 30-min periods. Since lithocholic acid-induced cholestasis was not reversible in our experiments, we could not infuse other bile acids after cholestasis with lithocholic acid had been established. In fact, lithocholic acid practically abolished bile flow after infusion as shown in Figure 3a. In contrast, 7ξ-methyl-LA did not decrease bile flow when infused at the same rate as lithocholic acid. In previous reports, it has been shown that cholestasis with lithocholic acid is not accompanied by a decreased bile acid excretion (2,6). However, in the present experiments bile acid excretion decreased dramatically because of the complete diminution of bile flow. 7ξ-Methyl-LA was secreted in bile without a decrease in bile flow even during high infusion rates. It comprised 33-75% of total bile acid as determined by GLC. In addition, 7ξ-methyl-LA did not affect the endogenous bile acid excretion.

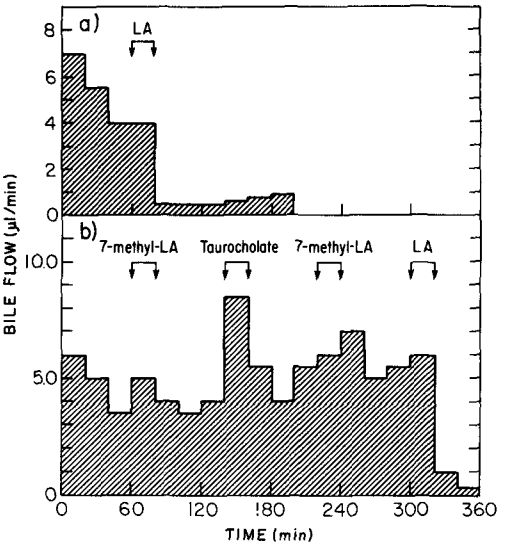

FIG. 3. Bile flow in a hamster receiving lithocholic acid (LA) (a), and a hamster receiving lithocholic acid (LA), 7ξ-methyl-LA, and sodium taurocholate (b).

Possible mechanisms of cholestasis produced by lithocholic acid have been suggested (5-9, 13-16). Presumably, lithocholic acid modifies the molecular organization of liver plasma membrane, including its binding to the canalicular membrane, thus inducing cholestasis. It also has been reported that the extent of the conversion to other bile acids may play a role in prevention of cholestasis on the basis of the results obtained in hamsters and rats (9). In our study, 7ξ-methyl-LA was not converted to other bile acids, and no cholestatic effect was observed. The deleterious effects of lithocholic acid have been examined in its relationship to the structure of the cholesterol molecule. Both lithocholic acid and cholesterol have a hydroxy

group at the 3-position. Obviously, 7ξ-methyl-LA, which possesses an additional methyl group at the 7-position, is structurally very different from lithocholic acid and cholesterol. This may explain in part its inability to induce cholestasis.

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Effects of Different Dietary Intake of Essential Fatty Acids on C20:3ω6 and C20:4ω6 Serum Levels in Human Adults

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ABSTRACT

Four diets which differed in fatty acid composition were provided for five months each to a group of 24 healthy nun volunteers. The diets contained 54% carbohydrates, 16% proteins and 30% lipids. One-third of the lipid part remained unchanged during the whole study, and two-thirds were modified during each period. For this latter portion, one of the following dietary fats was used: sunflower oil, peanut oil, low erucic acid rapeseed (LEAR) oil or milk fats. This procedure allowed an evaluation of the effects of various amounts of dietary linoleic acid (C18:2ω6) and alpha-linolenic acid (C18:3ω3) on the serum level of their metabolites. A diet providing a large amount of linoleic acid (14% of the total caloric intake) resulted in low levels of dihomo-gamma-linolenic acid (C20:3ω6) and arachidonic acid (C20:4ω6) in serum phospholipids and cholesteryl esters. A diet providing a small amount of linoleic acid (0.6% to 1.3% of the total caloric intake) induced high levels of ω6 fatty acid derivatives. Intermediate serum levels of C20:3ω6 and C20:4ω6 were found with a linoleic acid supply of about 6.5% of the total caloric intake. Serum levels of ω6 metabolites were not different after two diets providing a similar supply of C18:2ω6 (4.5% to 6.5% of the total caloric intake), although in one of them the supply of C18:3ω3 was higher (1.5% for LEAR oil versus 0.13% for peanut oil).

Under our experimental conditions (healthy human adults fed on a normo-caloric diet with 30% lipids), we tried to determine PUFA (linoleic and linolenic acid) allowances which should be recommended for adults. The aim of the study was to obtain a hypocholesterolemic or normocholesterolemic effect while keeping normal 20:3ω6 and 20:4ω6 serum levels which would evidence a normal linoleic acid metabolism. The amounts recommended are: linoleic acid 5 to 6% of the total calories; alpha-linolenic acid 0.5 to 1% of the total calories.

Lipids 20:227-233, 1985.

INTRODUCTION

The concept of essential fatty acid (EFA) deficiency, established as early as 1930 (1), has been amply confirmed (2,3). Nevertheless, human EFA requirements have not yet been well defined. The recommended amount of dietary linoleic acid (C18:2ω6) varies from 1-2% of the caloric intake (4) up to 15% (5). Between these two limits, various recommendations can be found (3,6). In "The Recommended Dietary Allowances" (1980), a maximum supply of 10% for polyunsaturated fatty acids (7) was suggested.

The recommended intake for alpha-linolenic acid (C18:3ω3) is generally 0.5% of total calories (8,9). However, recent studies have shown that some populations consume more of this fatty acid, with some benefit in the prevention of thrombosis and ischemic disease (10-12).

Δ6-Desaturase represents one of the most important enzymes in fatty acid metabolism, especially for the ω6 and ω3 series. It has been shown (13) that the preferential substrates for Δ6-desaturase were, with decreasing affinity, alpha-linolenic acid, linoleic acid and oleic acid. In order to study the competitive metabolism of linoleic and alpha-linolenic acids (precursors of eicosanoids), we tested four diets in which the amounts of fats remained unchanged (30% of the total caloric intake), but in which the fats differed qualitatively in each diet because of their fatty acid composition. Particularly, these diets provided different amounts of essential fatty acids. The study was carried out on healthy volunteers, living in a monastery, allowing a very stable, controlled diet for four periods of 5 months. This ensured a satisfactory metabolic balance. The composition and the metabolism of serum lipoproteins of the same subjects have been published previously (14).

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MATERIALS AND METHODS

Population

Before the study began the subjects were 69 Benedictine nuns strictly following dietary instructions (informed consent had been obtained from the subjects). At the beginning of the study the mean age was 46 ± 11 years (range 26 to 70). Subjects having metabolic disturbances (diabetes, hypercholesterolemia above 300 mg/100 ml, hypertriglyceridemia above 200 mg/100 ml) were not included. Selected subjects were divided into six age

groups (26-33, 34-41, 42-48, 49-55, 56-63, 64-70); in each group, four subjects were chosen at random. Plasma fatty acid analyses described in "Lipid analyses" section were made on these 24 women at the end of each dietary period.

Caloric intake and nutrient distribution remained constant during the whole study. The average total caloric intake was 2100 Kcal/day; the dietary composition was 16% proteins (2/3 of vegetable origin), 30% lipids and 54% carbohydrates (38% of which was starch). Two-thirds of the lipids (e.g., 20% of the total energy intake) were composed of one of the tested fats (vegetable or milk fats). The remaining lipids (10% of the total energy) were constant during the study and represented the food structural fats. Four diets were given, each for 5 months, containing one of the following dietary fats: sunflower oil, peanut oil, low erucic acid rapeseed (LEAR) oil and milk fats (butter and cream). The fatty acid composition of the dietary fats is shown in Table 1. The daily intake of linoleic acid, alpha-linolenic acid and cholesterol for each period is shown in Table 2.

TABLE 1

Fatty Acid Composition of the Tested Fats (weight %)

Fatty acids	Sunflower oil	Peanut oil	LEAR oil	Milk fats
4:0				3.4
6:0				1.9
8:0				1.26
10:0				2.89
12:0		0.17		3.62
14:0		0.85		11.8
14:1				0.36
16:0	6.19	11.50	5.40	32.61
16:1	0.09	0.10	0.30	2.5
18:0	4.74	3.60	1.60	12.47
18:1	16.96	41.50	56.30	24.9
18:2ω6	70.61	35.64	25.00	0.64
18:3ω3	0.20	0.20	8.40	1.51
20:0	0.30	1.50	0.60	
20:1	0.17	1.10	1.30	
22:0		3.20	0.40	
22:1		0.01	0.50	
22:2				
24		1.5	0.20	
P/S	6.3	1.68	4.17	0.03

The sunflower oil period was characterized by a large intake of linoleic acid (30.7 g/day/person or 13.7% of the total energy intake), a small intake of saturated fatty acids (8%), and a very small amount of alpha-linolenic acid (0.13%). The peanut oil period supplied an intermediate amount of linoleic acid (14.6 g/day/person, or 6.5% of the total energy), and a small amount of saturated fatty acid (8.3%).

The LEAR oil period provided an intermediate intake of linoleic acid (10.7 g/day/person, or 4.5% of the total energy), and a very small intake of saturated fatty acids (7.8%). It supplied a relatively large amount of alpha-linolenic acid (3.6 g/day/person or 1.5% of the total energy). During these last two vegetable oil periods (peanut, LEAR), the amount of polyunsaturated fatty acids stayed the same (about 6.5%). The fourth period (milk fats) provided a very small amount of linoleic acid (1.3 g/day/person or 0.6% of the total energy), and a large intake of saturated fats (22.5%), with a relatively high level of short and medium chain fatty acids (about 8 g/day/person). Moreover, this period provided more dietary cholesterol (400 mg/day/person) than other periods (about 290 mg/day/person).

Lipid Analyses

Analyses were made at the end of each 5-month period. Blood samples were collected by venous puncture of the cubital vein after 12 hr fasting. The serum was kept at 4 C and the following analyses were made the next day:

TABLE 2

Daily Intake of Saturated Fatty Acids, Linoleic Acid, Alpha-linolenic Acid and Cholesterol in the Four Tested Diets (g/day/person and % of total calories)

Diets	Saturated fatty acids		Linoleic acid		Alpha-linolenic acid		Cholesterol
	g/day/person	% cal.	g/day/person	% cal.	g/day/person	% cal.	mg/day/person
Sunflower oil	18.5	8	30.7	13.7	0.3	0.13	290
Peanut oil	19.3	8.3	14.6	6.5	0.3	0.13	290
LEAR oil	18.2	7.8	10.7	4.5	3.6	1.5	290
Milk fats	52.5[a]	22.5	1.3	0.6	2	0.9	400

[a]Of which 8 g/day/person and 3.5% of total calories were from short and medium chain saturated fatty acids.

total cholesterol according to Etienne et al. (15), and total triglycerides according to the glycerol 3-phosphate oxidase method (16).

Fatty acids of the serum lipid fractions were analyzed by the following method: Serum lipids were extracted with chloroform/methanol (2/1, v/v) according to Folch (17). Lipids were then separated by thin-layer chromatography on a silica gel plate; the solvent was a mixture of petroleum ether/diethyl ether/acetic acid (90/30/1, v/v/v). Each lipid fraction was identified after staining with rhodamine and comparison with known standards. Heptadecanoic acid was added to each collected fraction as an internal standard, and these fractions were transmethylated with a methanol/benzene/sulfuric acid solution (100/0.2/2, v/v/v). The fatty acid methyl esters were extracted using a heptane/water system. The fatty acid methyl ester spectra were analyzed on a Hewlett-Packard 5580 A gas chromatograph using a temperature program of 1 C/mn from 160 to 190 C. The injector was a Ros type (18); its temperature was 250 C. The auxiliary gases were hydrogen and air. The carrier gas was helium. A capillary column of 25 meters was used; its stationary phase was butane-diol succinate. The peak areas were measured by a Hewlett-Packard 338 A Integral Recorder.

Statistical Methods

The T-test (Student) was used for comparison of the average values and the F-test for the variance analysis.

RESULTS

Body weight remained constant during the four dietary periods (Table 3). Total serum cholesterol was significantly different after the four diets; the highest level was observed after the milk fat period, and the lowest after the sunflower period (Table 3). Serum triglycerides were not significantly different (Table 3). The amounts of fatty acids of the three serum lipid fractions (phospholipids, cholesteryl esters and triglycerides) were not different after the four periods. The fatty acid compositions of these serum lipid fractions are shown in Tables 4, 5 and 6. The phospholipid fatty acid composition changed with each diet (Table 4). After the LEAR and peanut oil periods, the composition of the $\omega 6$ family was similar. Though the relative percent of linoleic acid was high after sunflower oil and low after milk fats, the inverse proportions were observed for dihomo-gamma-linolenic acid (C20:3ω6) and arachidonic acid (C20:4ω6). The proportions of these two fatty acids were significantly lower after the sunflower oil (compared with the three other periods) and were significantly higher after the milk fats (compared with the 3 other periods). The proportions of alpha-linolenic acid (C18:3ω3) and of eicosapentaenoic acid (C20:5ω3) were highest after the LEAR diet and lowest after the sunflower and peanut oil diets. The relative percent of docosahexaenoic acid (C22:6ω3) was slightly lower after the sunflower oil period compared with the others, but this difference was not statistically significant. The proportions of myristic acid (C14:0) and stearic acid (C18:0) were statistically significantly higher after the sunflower oil than after the 3 other diets. The percent of palmitoleic acid (C16:1ω7) was higher (statistically significant) after the sun-

TABLE 3

Body Weight, Serum Total Cholesterol and Triglycerides After Each Period of Test Diet (mean ± S.D.)

Diets	Number of subjects	Body weight kg	Serum total cholesterol (mg/100 ml)	Serum triglycerides (mg/100 ml)
Sunflower oil	24	55.8 ± 6.9	175 ± 23 (b)	60 ± 33
Peanut oil	22	55.9 ± 6.5	205 ± 29 (a, b)	61 ± 44
LEAR oil	21	56.1 ± 6.7	190 ± 29 (b)	54 ± 34
Milk fats	23	55.1 ± 6.3	243 ± 40 (a)	69 ± 41
F-test		N.S.	$p < 0.05$	N.S.

N.S., not significant.

Significance of the average differences (T-test), comparison with the sunflower oil period : a : $p < 0.001$; comparison with the milk fat period : b : $p < 0.01$.

TABLE 4

Relative Percent of Fatty Acids in Serum Phospholipids (mean ± S.D.)

Fatty acids	Sunflower oil (n = 23)	Milk fats (n = 23)	LEAR oil (n = 21)	Peanut oil (n = 22)
14:0	0.73 ± 0.37 (c′)	0.37 ± 0.14 (c)	0.31 ± 0.14 (c)	0.24 ± 0.16 (c) (b′)
16:0	28.3 ± 2.8	30.4 ± 3.5	26.7 ± 2.6	26.0 ± 1.4
16:1ω7	0.81 ± 0.35	0.68 ± 0.37	0.19 ± 0.15 (c) (c′)	0.12 ± 0.1 (c) (c′)
18:0	19.8 ± 1.8 (c′)	16.0 ± 1.5 (c)	14.6 ± 1.2 (c) (c′)	14.8 ± 0.9 (c) (b′)
18:1ω9	8.7 ± 1.1	10.2 ± 1.4	13.5 ± 1.9	11.6 ± 0.8
18:2ω6	21.6 ± 4 (c′)	16.3 ± 2.3 (c)	22.4 ± 2.6 (a) (a′)	24.3 ± 2.9 (b′)
18:3ω6	–	–	–	–
18:3ω3	–	0.16 ± 0.08	0.30 ± 0.15	0.09 ± 0.05
20:3ω6	1.97 ± 0.6 (c′)	3.97 ± 0.9 (c)	2.83 ± 0.7 (c) (c′)	3.33 ± 0.8 (c)
20:4ω6	8.9 ± 2.3 (c′)	12.22 ± 1.7 (c)	10.5 ± 2.3 (a) (b′)	11.11 ± 1.8 (b) (a′)
20:5ω3	0.8 ± 0.7 (c′)	2.08 ± 0.9 (c)	2.01 ± 1.9 (c) (d)	0.8 ± 0.24 (c′)
22:6ω3	4.02 ± 2.1	5.57 ± 2.3	6.57 ± 1.80	6.10 ± 2.45

Comparison by T-test, with sunflower oil period, a = $p < 0.05$, b = $p < 0.01$, c = $p < 0.001$; with milk fat period, a′ = $p < 0.05$, b′ = $p < 0.01$, c′ = $p < 0.001$; with peanut oil period, d = $p < 0.01$.

flower and the milk fat diets than after the LEAR oil and peanut oil diets. In cholesteryl esters, the same differences in the fatty acid composition according to the dietary fat were found (Table 5). More linoleic acid and less alpha-linolenic, dihomo-gamma-linolenic, arachidonic and eicosapentaenoic acids after the sunflower oil diet compared with the other periods. In the triglyceride fraction (Table 6), the level of the different fatty acids was correlated with the dietary lipids of each period. Thus, after the milk fat period, higher proportions of saturated fatty acids were observed. After the LEAR and peanut diets, there was a higher proportion of oleic acid. After the sunflower diet, a higher proportion of linoleic acid appeared. With the latter diet, there was more than 33% linoleic acid in the serum triglycerides.

DISCUSSION

According to these results, there is a hypercholesterolemic effect of the milk fats compared to the other three diets, or a hypocholesterolemic effect of the polyunsaturated fatty acid-rich diets. This already has been described in various experimental (19,20) or epidemiological studies (21,22). The high levels of serum cholesterol observed after the milk fat diet could be due to the great amount of dietary saturated fatty acids (particularly long chain fatty acids) and/or the consequence of the dietary amount of cholesterol, which was higher during the milk fat period. The protocol of this study does not allow a conclusion on this point. The three diets which provided a small amount of saturated fatty acids (sunflower oil, LEAR oil and peanut oil), resulted in similar serum cholesterol levels (175 to 205 dg/100 ml). Since the dietary amount of polyunsaturated fatty acids was different during these three periods (31 g/day for the sunflower oil diet, 15 g/day for the peanut oil diet, and 14.3 g/day for the LEAR oil diet), it did not appear to play an important role in controlling the serum cholesterol level. For this reason, a polyunsaturated fatty acid intake higher than the recommended one (6.5% of the total energy) does not seem indicated.

The analyses of serum phospholipid and cholesterol ester fatty acids show important differences depending on the dietary fats. The different supplies of PUFA seem responsible for different serum levels of dihomo-gamma-

TABLE 5

Relative Percent of Fatty Acids of Serum Cholesteryl Esters (mean ± S.D.)

Fatty acids	Sunflower oil (n = 23)	Milk fats (n = 23)	LEAR oil (n = 21)	Peanut oil (n = 22)
14:0	0.54 ± 0.21 (c′)	1.31 ± 0.39 (c)	0.9 ± 0,57 (b) (b′)	0.50 ± 0.22 (c′)
16:0	9.44 ± 1.09	12.3 ± 0.95	9.41 ± 1	10.3 ± 0.9
16:1ω7	2.26 ± 0.93 (b′)	4.86 ± 1.2 (b)	2.97 ± 1.91 (c′)	2.09 ± 1.03 (c′)
18:0	0.84 ± 0.21	0.94 ± 0.21	0.57 ± 0.09	0.79 ± 0.19
18:1ω9	11.66 ± 2.7 (c′)	20.6 ± 2.23 (c)	19.1 ± 1.55 (c)	17.99 ± 1.37 (c) (c′)
18:2ω6	66.9 ± 5.77 (c′)	47 ± 3.88 (c)	55.7 ± 4.2 (a)	58.1 ± 4.18 (a) (b′)
18:3ω6	0.62 ± 0.46	0.84 ± 0.39	0.63 ± 0.28	0.82 ± 0.32
18:3ω3	0.02 ± 0.03 (c′)	0.67 ± 0.22 (c)	1.20 ± 0.32 (c)	0.27 ± 0.12 (c) (c′)
20:3ω6	0.47 ± 0.27 (c′)	0.73 ± 0.17 (c)	0.53 ± 0.14 (c′)	0.68 ± 0.19 (b)
20:4ω6	6.19 ± 2.3 (b′)	7.71 ± 1.17 (b)	6.79 ± 1.4 (a′) (d)	6.99 ± 1.36
20:5ω3	0.03 ± 0.11 (c′)	1.93 ± 0.78 (c)	1.20 ± 0.36 (c) (c′)(e)	0.71 ± 0.18 (c) (c′)
22:6ω3	0.72 ± 0.35	1.04 ± 0.54	0.99 ± 0.40 (d)	0.77 ± 0.28

Comparison by T-test, with sunflower oil period, a = $p < 0.05$, b = $p < 0.01$, c = $p < 0.01$; with milk fat period, a′ = $p < 0.5$, b′ = $p < 0.01$, c′ = $p < 0.001$; with peanut oil period, d = $p < 0.05$, e = $p < 0.01$.

linolenic, arachidonic, eicosapentaenoic and docosahexaenoic acids. The peanut oil diet supplied 14.9 g/day/person PUFA (containing 14.6 g linoleic acid), which represented 6.5% of the total caloric intake. With this diet, the levels of dihomo-gamma-linolenic acid and arachidonic acid of the serum phospholipids were close to those found by Holman et al. (23) and by Zumanski et al. (24) in subjects having a standard food intake. We can consider that such levels correspond to a normal Δ6-desaturase activity. After the sunflower diet, which supplied 31 g/day/person PUFA (30.7 linoleic acid), which represented 13.7% of the total caloric intake, the lowest values of dihomo-gamma-linolenic and arachidonic acids were observed. These values were even lower than those observed with the milk fat diet (3.3 g/day/person PUFA containing 1.3 g linoleic acid). These observations with a high supply of linoleic acid confirm data obtained in other circumstances: in orally fed infants (25); in infants on TPN with fat emulsion, in which low levels of urinary prostaglandins were found (26) and in adults on TPN with fat emulsion (27). This effect also has been described in three animal species: in minipigs (28) showing a decrease in arachidonic acid in tissue phospholipids; in rats (29-32), and in rabbits where a decrease in the prostacyclin level was observed in the heart (32). Finally, a decrease in aorta phosphatidylinositol arachidonic acid and a decrease in prostacyclin formation also were reported in rabbits fed with linoleic acid at 11% of the caloric intake (33,34).

Does a high intake of linoleic acid inhibit the Δ6-desaturase activity (an important and limiting enzyme in the synthesis of the essential fatty acid metabolites (35,36)? The sunflower diet also supplied the lowest amounts of oleic acid and alpha-linolenic acid which are substrates of Δ6-desaturase. Thus, this diet leads to the weakest competition of oleic acid and linolenic acid as substrates of Δ6-desaturase with linoleic acid, and should lead to the highest levels of linoleic acid derivatives. On the other hand, the low levels of dihomo-gamma-linolenic acid and arachidonic acid also could be due to a competition of the fatty acids for the position 2 of the phospholipids, the high

TABLE 6

Relative Percent of Fatty Acids of Serum Triglycerides (mean ± S.D.)

Fatty acids	Sunflower oil (n = 23)	Milk fats (n = 23)	LEAR oil (n = 21)	Peanut oil (n = 22)
14:0	1.56 ± 0.48 (c′)	2.71 ± 1.22 (c′)	1.39 ± 0.68 (c′)	1.43 ± 0.51 (c′)
16:0	21.9 ± 5.23 (c′)	29.4 ± 2.38 (c)	19.27 ± 2.24 (b) (c′)	22.67 ± 2.51 (b) (c′)
16:1ω7	4.30 ± 1.33	4.84 ± 1.49	4.57 ± 1.63	3.86 ± 1.44 (b′)
18:0	3.52 ± 0.65 (c′)	4.99 ± 0.98 (c)	3.26 ± 0.8 (c′)	3.54 ± 0.6 (c′)
18:1ω9	29.63 ± 5.06 (c′)	40.1 ± 3.77 (c)	44.54 ± 2.07 (c) (c′)	41.45 ± 2.36 (c)
18:2ω6	34.93 ± 7.04 (c′)	12.94 ± 2.44 (c)	20.83 ± 2.39 (c) (c′)	22.9 ± 2.98 (c) (c′)
18:3ω6	0.90 ± 0.40 (c′)	0.20 ± 0.22 (c)	0.44 ± 0.25 (c) (b′)	0.63 ± 0.25 (c) (c′)
18:3ω3	0.83 ± 0.39	0.79 ± 0.20	2.47 ± 0.62 (c) (c′)	0.63 ± 0.22
20:3ω6	0.28 ± 0.38	0.24 ± 0.13	0.25 ± 0.11	0.38 ± 0.3
20:4ω6	1.64 ± 0.52	1.67 ± 0.39	1.39 ± 0.29 (a) (a′)	1.42 ± 0.67 (a′)
20:5ω3	0.01 ± 0.06 (c′)	0.48 ± 0.32 (c)	0.33 ± 0.17 (c) (a′)	0.11 ± 0.30 (c′)
22:6ω3	–	–	–	–

Comparison by T-test, with sunflower oil period, a = $p < 0.05$, b = $p < 0.01$, c = $p < 0.001$; with milk fat period, a′ = $p < 0.05$, b′ = $p < 0.01$, c′ = $p < 0.001$.

level of linoleic acid preventing the esterification by arachidonic (or dihomo-gamma-linolenic) acid. Moreover, more than one-third of the triglyceride fatty acids is linoleic acid. A part of this could be in position 1 or 3 of the triglycerides.

The milk fat diet supplied 1.3 g/day/person of linoleic acid, which represents 0.6% of the total caloric intake. This diet resulted in a low level of linoleic acid, but in high levels of dihomo-gamma-linolenic and arachidonic acids in serum phospholipids. Thus, the ingestion of 52.5 g/day/person saturated fatty acids by adults did not have a negative influence on Δ6-desaturation. Such a result was observed in infants fed 3 g/kg/day milk fat saturated fatty acids (6). The two groups with low or moderate intakes of linoleic acid (milk fat and LEAR oil) had significantly higher levels of cholesterol ester and phospholipid dihomo-gamma-linolenic acid ($p < 0.001$) than the group with a high intake of linoleic acid (sunflower group). Moreover, if the milk fat diet and the peanut oil diet groups are compared, phospholipid dihomo-gamma-linolenic levels are similar and only phospholipid arachidonic acid level is significantly lower ($p < 0.05$) in the peanut oil diet group. Δ6-Desaturase activity is thus not different in those two groups.

The LEAR oil diet is characterized by the most usually recommended linoleic acid intake for the overall population (10.7 g/day/person or 4.5% of the caloric intake) and a high alpha-linolenic acid intake (3.6 g/day/person or 1.5% of total caloric intake). Thus the linoleic acid/linolenic acid ratio is 3, instead of the usually found or recommended ratio of 10. In the LEAR oil group, the cholesterol ester eicosapentaenoic acid levels and the phospholipid eicosapentaenoic acid and docosahexaenoic acid levels were significantly higher than those found in the sunflower oil or peanut oil groups.

However, the LEAR oil and the peanut oil diets gave similar levels of dihomo-gamma-linolenic and arachidonic acids in phospholipids and cholesterol esters. Though the linoleic acid supply was similar in these two diets, the LEAR oil diet provided a large amount of alpha-linolenic acid (three times more than the recommended intake). Even in this case, there was no effect on the levels of linoleic acid metabolites. These findings indicate that a

linoleic acid/linolenic acid ratio of 3 with a linoleic intake of 10.7 g/day does not induce a competition between the ω6 and the ω3 families.

In conclusion, in human adults on a normocaloric diet and with a moderate amount of dietary lipids (30% of the total energy intake), we observed a decrease of the serum levels of linoleic acid derivatives when the dietary supply of linoleic acid was high (30.7 g/day or 13.7% of total caloric intake) (sunflower diet). This decrease was not found with a moderate linoleic acid intake (10.7 g/day or 4.5% of total caloric intake) and a linoleic acid/linolenic acid ratio of 3 (LEAR oil diet). This decrease was also not found with a low linoleic acid intake (1.3 g/day or 0.6% of total caloric intake), a high intake of milk fat saturated acids (52.5 g/day) and a linoleic acid/linolenic acid ratio of 0.65 (milk fat diet). Moreover, we did not observe any differences in blood total cholesterol and triglycerides after a diet providing 4.5% (LEAR oil diet) or 13.7% (sunflower oil diet) of the total caloric intake as linoleic acid.

A diet providing 1.5% of the total caloric intake as linolenic acid (LEAR oil diet) gave significantly higher levels of phospholipid eicosapentaenoic and docosahexaenoic acids. These levels of ω3 fatty acids could bring some benefit in the prevention of thromboses and ischemic disease (10-12). Finally, such a supply of alpha-linolenic acid did not induce a competition between the ω6 and ω3 families. Thus, we recommend the following intakes: linoleic acid, 5-6% of the total caloric intake; linolenic acid, 0.5-1% of the total caloric intake.

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METHODS

Synthesis of Octadecynoic Acids and [1-^{14}C] Labeled Isomers of Octadecenoic Acids

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ABSTRACT

Geometric and positional isomers of [1-^{14}C] octadecenoic acids have been synthesized by modifications of published procedures. Positional isomers of octadecynoic acids also have been synthesized to obtain the geometric and positional isomers of the unlabeled octadecenoic acid analogs. The syntheses were accomplished by coupling a haloalkyl compound with a substituted acetylene using n-butyl lithium in hexamethylphosphoramide. The coupled product, either a 17- or 18-carbon acetylenic alcohol, could be semihydrogenated and chain extended to afford a carboxy labeled derivative, could be partially hydrogenated and chain extended to afford a carboxyl labeled *cis*- or *trans*-octadecenoic acid in the former case. In the latter case, octadecynoic, *cis*-octadecenoic or *trans*-octadecenoic acids could be obtained by the appropriate reactions. The methods used in this study enabled the synthesis of ^{14}C-labeled fatty acids in generally higher yields and by simpler reactions than were previously possible.

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INTRODUCTION

Both the position and geometry of a double bond in an unsaturated fatty acid exert profound effects on metabolic reactions. The need in our laboratory for a series of labeled [1-^{14}C] and unlabeled octadecenoic fatty acids to facilitate comparison of rates of reaction of isomers in several enzyme systems required syntheses of these acids.

The series of labeled isomeric *trans* acids was used for the study of chain elongation (1) and desaturation (2) by rat liver microsomes. The series of labeled *cis* isomers was similarly used in the study of desaturation (3). The unlabeled series of *trans* (4) and *cis* (5) isomers has been used to study inhibition of Δ6-, Δ5- and Δ9-desaturases. The unlabeled series also has been used to study relative rates of β-oxidation of positional isomers by heart mitochondria (6). The comparative studies on these several systems have been summarized (7,8). The general outline of the synthesis of these compounds has been given orally and summarized briefly (9), but the details of the synthesis have not been published previously. The syntheses were based on modifications of published procedures incorporating several new techniques resulting in increased yield and ease of preparation. The preparations of all *cis*- and *trans*-octadecenoic fatty acids as well as their monoacetylenic precursors have been described (10,11) and reviewed (12), but the syntheses described here permit [1-^{14}C] labeling and are of necessity different from the published methods leading to unlabeled products.

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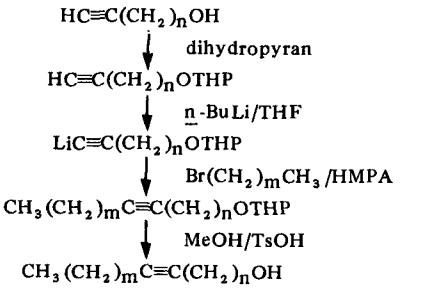
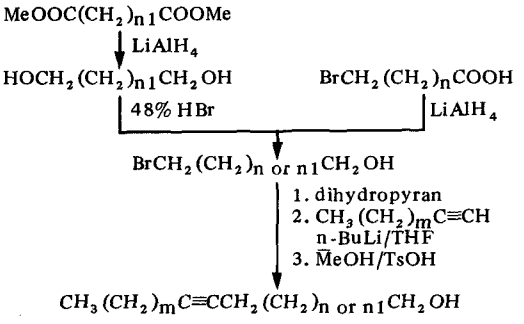
PROCEDURAL APPROACH

Depending upon the position of the *cis*- or *trans*-double bond in the desired unsaturated fatty acid, the synthesis of a [1-^{14}C] labeled octadecenoic acid was accomplished by one of two synthetic schemes. As seen in Figure 1, an ω-alkynyltetrahydropyranyl ether was condensed with an alkyl halide, and in Figure 2, an ω-halotetrahydropyranyl ether was condensed with a terminal alkyne. In both cases, the reactants were chosen so that, upon hydrolysis



Triple bond position:	2	3	4	5	11
m	13	12	11	10	4
n	1	2	3	4	10
Compound number:			III		

FIG. 1. Synthesis of heptadecynols.



Triple bond position:	6	7	8	9	10	12	13	14[a]	15	16[a]
m	9	8	7	6	5	3	2	1	0	-1[b]
n	3	4	5	6		9				
n1					7		10	10	12	12
Compound number:	II									

[a]Extend hexadecynol with KCN, hydrolyze to acid and reduce to heptadecynol with $LiAlH_4$.

[b]The protected bromoalcohol is coupled with acetylene to produce the terminal hexadecynol.

FIG. 2. Synthesis of heptadecynols.

of the condensation product, a heptadecynol was obtained. The heptadecynol was then semihydrogenated to a *trans*-alkenol with lithium/liquid ammonia (11) or semihydrogenated with "P-2 Nickel" catalyst poisoned with ethylene diamine (EDA) (13) to yield the *cis*-isomer. To prepare a [1-^{14}C] labeled octadecenoic fatty acid, the heptadecenol was converted to a mesylate, which was then extended with K^{14}CN (14), and hydrolysis of the resulting nitrile yielded the desired [1-^{14}C] octadecenoic acid (Fig. 3). Howling et al. (15) have prepared several [1-^{14}C] labeled octadecenoic acids by the mesylate-cyanide method.

The corresponding unlabeled octadecenoic acids, with the exception of the 9- and 11-isomers, were prepared by first coupling the appropriate constituents and hydrolyzing the resulting tetrahydropyranyl ether to yield an octadecynol. The octadecynol was oxidized to the corresponding octadecynal with dipyridine-chromic anhydride complex (16) followed by further oxidation with silver nitrate and sodium hydroxide (17) to the octadecynoic acid (Figs. 4 and 5). Verification of the location of the triple bond in the octadecynoic acids was made by their conversion to pyrrolidide derivatives followed by mass spectrometric analysis as described by Valicenti et al. (18). Reduction of the unlabeled octadecynoic acids with "P-2 Nickel"/ethylene diamine (13) or by lithium/liquid ammonia (11) produced the *cis*- or *trans*-octadecenoic acid, respectively. When these were purified, converted to the pyrrolidides and analyzed by mass spectrometry, they gave spectra equivalent to the published spectra of monoenoic C_{18} acids (19), verifying the expected structure in each case.

As an alternative approach, especially in the case of *cis* isomers, the appropriate octadecenol may be prepared from the octadecynol, which then may be oxidized to the corresponding octadecenal (16) followed by a further oxidation with silver nitrate and sodium hydroxide (17) to the octadecenoic acid.

The condensations were facilitated by using

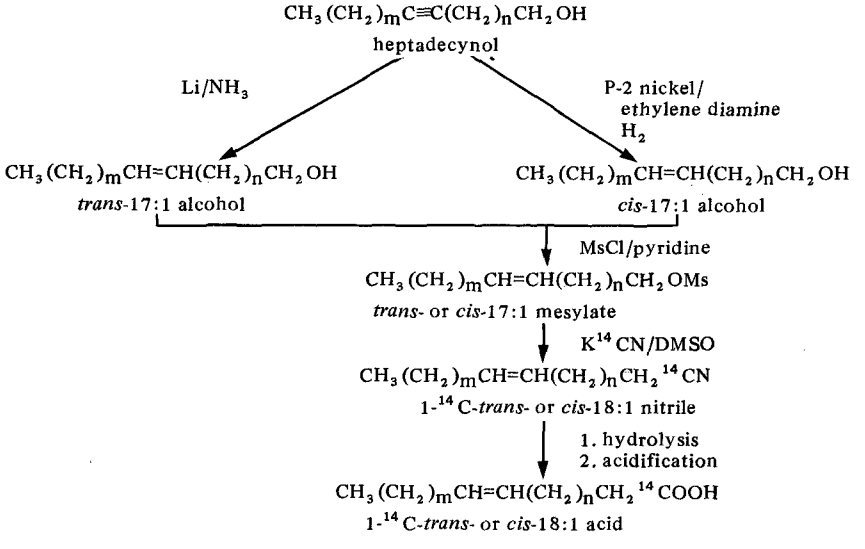
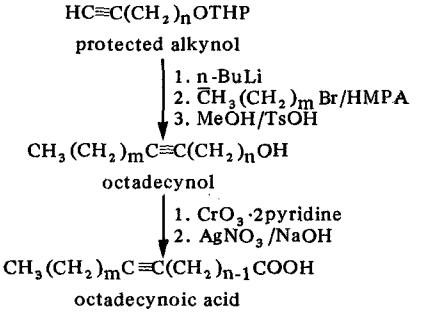


FIG. 3. Synthesis of 1-^{14}C-octadecenoic acids.

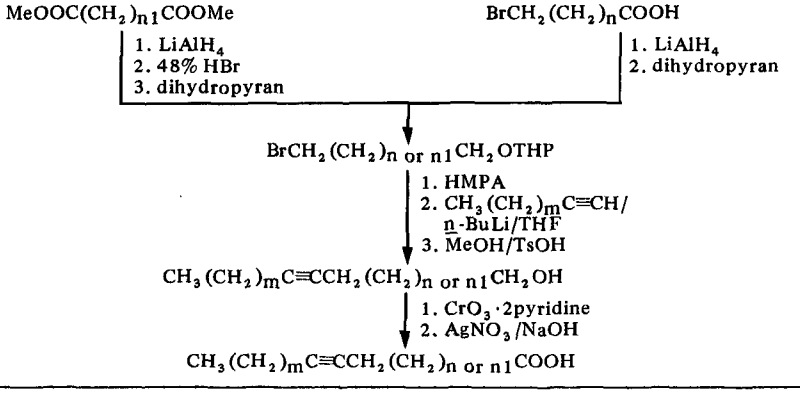


Triple bond position:	3	4	5	6	7	8
m	13	12	11	10	9	8
n	2	3	4	5	6	7
Compound number:		IV				

FIG. 4. Synthesis of octadecynoic acids.

hexamethylphosphoramide (HMPA) as a solvent yielding products in high yield. [Note that HMPA is a suspected carcinogen and that care must be exercised in its use.] The use of HMPA was based on the work of Normant (20), who suggested it as a powerful aprotic solvent in the formation of carbon-carbon linkages from alkyl halides and acetylide ions. Brattesani and Heathcock (21) used HMPA as a solvent in the preparation of simple internal alkynes. Beckman and coworkers (22) prepared lithium acetylide from n-butyl lithium and acetylene in tetrahydrofuran (THF), which was then reacted with alkyl halides in THF-HMPA to give long chain 1-alkynes. Gilman and Holland (23) synthesized a series of C_{10} to C_{14} monoynoic acids using this solvent, and Schwarz and Waters (24) reported the synthesis of acetylenic intermediates in almost quantitative yields for the study of insect sex attractants when they carried out coupling reactions in a mixture of THF-HMPA.

Commercially available materials were used to prepare the 2-, 3-, 4- and 5-heptadecynols. However, to prepare the 6- through 9- and 12-heptadecynols and the 12- and 13-octadecynols, the required ω-bromoalcohols were prepared by lithium aluminum hydride reduction of the corresponding bromoacids. The synthesis of the 10- and 13- through 16-heptadecynols and the 14- through 17-octadecynols involved the reduction of the methyl esters of the dibasic acids followed by conversion of the resulting α,ω-diols to ω-bromoalcohols according to the method of Pattison et al. (25). Dodec-11-ynol was synthesized to prepare the 11-isomer. The synthesis of the unlabeled 6-, 7- and 8-octadecynols necessitated the preparation of 6-heptyn-, 7-octyn- and 8-nonynol. These alkynols were prepared by converting α,ω-dichloro or -dibromoalkanes to iodochlorides or iodobromides with sodium iodide in acetone. These in turn were condensed with sodium acetylide to



Triple bond position:	10	12	13	14[a]	15	16[a]	17[b]
m	6	4	3	2	1	0	-1[c]
n		9	10				
n1	7			10	12	12	12
Compound number:		V					

[a]Extend heptadecynol with KCN and hydrolyze to octadecynoic acid.

[b]Extend hexadecynol with diethyl malonate, hydrolyze and decarboxylate to octadecynoic acid.

[c]The protected bromoalcohol is coupled with acetylene to produce the terminal hexadecynol.

FIG. 5. Synthesis of octadecynoic acids.

form an alkynyl chloride or bromide which was converted to an alkynyl iodide by reaction with sodium iodide in acetone. Finally, the alkynyl iodide was reacted with sodium acetate in acetic acid to form an alkynyl acetate. The acetate then was reduced to the corresponding alkynol with lithium aluminum hydride.

Because of the unavailability of appropriate starting materials (i.e. a bifunctional undecane or tridecane), the preparation of the 14- and 16-isomers involved a coupling reaction to give a hexadecynol, followed by elongation via mesylation and cyanation. The C_{17} nitrile was hydrolyzed to the acid and reduced with lithium aluminum hydride to furnish the desired heptadecynol.

The condensations in either of the above schemes were carried out by formation of the desired lithium salt of the alkyne using n-butyl lithium and condensation of this salt with a terminal bromoalcohol to produce the alkynol. Because this procedure employs lipophilic solvents at close to ambient temperature, the solubilities of intermediates longer than 10 carbons are greater than in the method using liquid ammonia (11). This effect and the limited degradation of the alkyl halide in the HMPA system are undoubtedly the chief reasons for the increased yield of condensation products in this step, ranging from 60 to 75%.

Where it was applicable, Barve and Gunstone (11) reduced several octadecynoic acids to the corresponding *trans*-octadecenoic acids using sodium or lithium in ammonia (26,27). In our case, to avoid the necessity of reducing radioactive octadecynoic acids, it was necessary to reduce the heptadecyn-1-ols to the *trans*-heptadecen-1-ols so that radioactive chain extension could be carried out as a last step. Complete reduction of the ynols was not always obtained using this procedure, most likely due to the low solubility of the substrate alcohol in liquid ammonia. In some cases, this necessitated a second reduction or separation of the alkenols from unreduced alkynols by argentation thin-layer or column chromatography. In contrast to the difficulty in producing *trans*-alkenols from acetylenic alcohols, reduction of several octadecynoic acids yielded products of 99% purity, presumably due to the higher solubility of the acid in liquid ammonia. Examples of the major steps in the synthesis are given in the following section.

EXPERIMENTAL

Reagents and Materials

Radioactive potassium cyanide was purchased from American Radioactive Chemical Corp., Sanford, Florida. n-Butyl lithium (Aldrich Chemical Co., Inc.) was a 1.6 M solution in hexane. Hexamethylphosphoramide (Fisher Scientific Co.) was distilled under reduced pressure (67 C/0.5 torr) from BaO and stored over Type 4A (Davison) molecular sieves (Fisher Scientific Co.). Dimethylsulfoxide (Fisher Scientific Co.) was dried over $CaSO_4$, distilled under reduced pressure (50 C/2-3 torr) from NaOH and stored over 4A molecular sieve. Pyridine (Fisher Scientific Co.) was dried over KOH pellets, distilled from BaO and stored over 4A molecular sieve. Tetrahydrofuran (Fisher Scientific Co.) was refluxed with $LiAlH_4$, distilled and stored over sodium ribbon before use. Methanesulfonyl chloride (Eastman Organic Chemicals) was redistilled (bp 61-62 C/18 torr). Ethylene diamine (Fisher Scientific Co.) was distilled from sodium and stored over 4A molecular sieve. Acetone was dried over 4A molecular sieve and distilled from $KMnO_4$. Ethyl ether (reagent grade absolute) was used as received. Petroleum ether (b.p. 30-60 C) was washed with concentrated sulfuric acid several times until the acidic phase was colorless. The hydrocarbon layer was washed with water, saturated sodium bicarbonate solution, dried over Drierite (W.A. Hammond Drierite Co.) and distilled. Methyl cellosolve was dried over $CaSO_4$ and distilled before use.

Alkynes, alkynols, alkyl halides, bromoacids, dicarboxylic acids and alkyl diols were of high purity and were used as received. ITLC™-SA chromatography paper (polysilicic acid gel impregnated glass fiber paper) was purchased from Gelman Instrument Co., Ann Arbor, Michigan. Gas liquid chromatographic (GLC) analyses were performed on a Packard Model 427 instrument equipped with a flame ionization detector (FID); the detector temperature was at 270 C, and the injector temperature at 250 C with helium as the carrier gas. To determine purity of starting materials and intermediates such as alkyl halides, alkynl halides, alkynols and products such as acetylenic or olefinic alcohols as their acetates, a 1/8" x 8' column of 10% EGSS-X on 100/120 mesh Gas Chrom P at 180 C was used while the analyses of the alkynoic or alkenoic acids as their methyl esters were carried out on a 1/8" x 8' column of 15% OV-275 on 100/120 mesh Gas Chrom Q at 230 C. Infrared (IR) spectra were determined on a Perkin-Elmer IR-21 instrument. Mass spectra (MS) were determined on a Hitachi Perkin-Elmer RMU-6D instrument. Melting points (uncorrected) were determined on a Reichert (Kofler) apparatus. Screw cap culture tubes (16 x 75 mm) having Teflon-lined caps were used for the radioactive synthesis and were tested against leakage before use.

The following are examples of the synthetic steps employed in the synthesis of the [1-^{14}C] octadecenoic acid isomers and the unlabeled octadecenoic acid isomers.

Protection of the Hydroxy Group (Fig. 1)

2-(4-Pentynyloxy)-tetrahydropyran (compound I). Into a 500-ml flask equipped with a magnetic stirrer was placed 10.3 g (122 mmol) 4-pentynol dissolved in 200 ml ether to which was added 15.0 g (180 mmol) dihydropyran and 600 mg p-toluenesulfonic acid. The reaction mixture was stirred at room temperature for several hours after which time thin-layer chromatographic (TLC) analysis, petroleum ether/ether/acetic acid (80:20:1, v/v/v), indicated good conversion to the ether. The reaction mixture was poured into aqueous 10% sodium hydroxide, and the ether phase was washed twice with water and dried over anhydrous sodium sulfate. The crude protected alkynol upon removal of solvent yielded 22.6 g which was then distilled through a small, packed column. A small forerun was discarded and the fraction boiling at 46-50 C/0.3 torr was collected to yield 13.85 g (66.3%) of compound I.

Coupling of a Protected Halo-Alcohol with a Terminal Alkyne and Removal of the Protecting Group (Fig. 2)

7-Heptadecynol (II). To 3.8 g (25 mmol) 1-undecyne in 20 ml freshly distilled THF in a 100-ml 3-necked flask equipped with a magnetic stirrer, a thermometer, a pressure equalized dropping funnel, an argon inlet and an outlet from the top of the dropping funnel to a bubbler, was added dropwise 20 ml (32 mmol) of 1.6 M n-butyl lithium in hexane at such a rate that the temperature did not rise above 10 C with cooling using an ice bath. Then 6.0 g (22.6 mmol) of the tetrahydropyranyl ether of 6-bromohexanol in 35 ml HMPA was added dropwise at such a rate as to keep the temperature below 25 C during the addition. The ice bath was removed and stirring continued for 1 hr at room temperature. Water was added to the reaction mixture, and the products were extracted with ether. The organic phase was washed with saturated sodium bicarbonate and with water and then dried over sodium sulfate. TLC, using petroleum ether/ether (90:10, v/v) indicated good conversion to the coupled product, which was purified by preparatory TLC on eight 3 mm Silica Gel H plates to give 5.1 g (56%) of the acetylenic tetrahydropyranyl ether. The ether was hydrolyzed by refluxing with ca. 250 mg of p-toluenesulfonic acid in methanol on the steam bath for several hours. After removal of the methanol on a rotary evaporator, the residue was dissolved in ether, washed with saturated sodium bicarbonate, water and dried over magnesium sulfate. Removal of solvent gave 3.7 g of acetylenic alcohol. Preparatory TLC, petroleum ether/ether (60:40, v/v), on 3 mm plates gave 3.45 g (60.5%) of II with a melting point of 24.5-26 C which was found to be a single substance by TLC analysis and by GLC of the acetate.

Coupling of a Protected Alkynyl Alcohol with an Alkyl Halide and Removal of the Protecting Group (Fig. 1)

4-Heptadecynol (III). Compound III was prepared in a manner similar to compound II. To a solution of 5.0 g (29.7 mmol) of the THP ether of 3-butynol in 30 ml of THF was added dropwise 21.5 ml (35 mmol) of 1.6 M n-butyl lithium in hexane. After 5 min, 7.7 g (31 mmol) of 1-bromodecane in 50 ml HMPA was added, and the reaction mixture was stirred at room temperature for 3 hr. The crude protected alcohol was isolated from the reaction mixture as above and purified on twelve 3-mm Silica Gel H plates to give 6.89 g (60%) of the THP ether of III. The protecting group was removed, and the oil was dissolved in 50 ml of petroleum ether and recrystallized in the freezer, yielding 3.9 g (52%) of III as white crystals melting at 34-35 C. TLC analysis using petroleum ether/ether (60:40, v/v) indicated a single compound.

Preparation of an Alkynyl Alcohol from a Dihalide

11-Dodecynol. In a 500-ml flask equipped with a magnetic stirrer and reflux condenser was placed 68.4 g (456 mmol) sodium iodide in 255 ml dry acetone. After the sodium iodide had dissolved, 80 g (380 mmol) 1,10-dichlorodecane was added. The mixture was refluxed for 9 hr. After the solution had cooled, the insoluble sodium chloride was removed by filtration and washed well with ether, and the ether wash was added to the acetone solution. Most of the solvent was removed on a rotary evaporator. The residue was dissolved in ether, washed with saturated sodium chloride solution and water and dried over magnesium sulfate. Removal of the solvent gave 113.0 g of red oil which was distilled through a 1.5 × 13.5 cm column containing protruded packing and gave 58.0 g (50.5%) of a colorless liquid boiling at 121-124 C/0.2 torr. This was judged to be > 99.9% pure 1-chloro-10-iododecane by GLC analysis. The IR spectrum (film) revealed absorbance of 3267 (s), 2100 (w), 725 (s) and 650 (s).

Gaseous ammonia was condensed into a 500 ml 3-necked flask equipped with a dry ice condenser and gas dispersion tube until 200 ml of liquid was obtained. The liquid ammonia was

kept in the liquid state with a dry ice-isopropyl alcohol bath. Into this was bubbled acetylene gas (dried by passage through concentrated sulfuric acid) until the ammonia was saturated. While continuing to add acetylene to the liquid ammonia, 4.6 g (176 mmol) of sodium metal was added in small pieces and the solution was stirred for 30 min. With stirring, 53.5 g (176 mmol) 1-chloro-10-iododecane in 50 ml tetrahydrofuran was added. The dry ice condenser was kept in place for 1 hr and then removed, and the reaction mixture was allowed to warm to room temperature overnight. The excess THF was removed under a stream of nitrogen. The residue was carefully neutralized by adding saturated ammonium chloride solution. The aqueous mixture was then extracted with 500 ml petroleum ether and dried over anhydrous magnesium sulfate. After removal of the solvent under vacuum, the crude material (35.3 g) was distilled. The fractions boiling at 80-107 C/0.3-1.5 torr were combined, yielding 23.7 g (67%) 12-chlorododecyne.

To 92.3 g (610 mmol) sodium iodide in 200 ml dry acetone in a 500 ml flask, equipped with a magnetic stirrer and reflux condenser, was added 20.2 g (101 mmol) 12-chlorododecyne. The mixture was refluxed overnight with stirring. After cooling, the acetone was removed on a rotary evaporator, 300 ml water was added and the product was extracted with 600 ml ether. The ether layer was washed once with 10% sodium thiosulfate and twice with water. After drying over sodium sulfate and removal of solvent, 31 g pale yellow oil was obtained. Application of heat and vacuum produced a pot residue of 28 g (94%) which GLC indicated to be pure 12-iodododecyne. IR (film): 3267 (s), 2100 (w), 1720 (s), 650 (s).

To 15.2 g (190 mmol) sodium acetate in 22.8 (380 mmol) acetic acid was added 28 g (95 mmol) 12-iodododecyne, and the mixture was refluxed overnight. After the reaction mixture had cooled, it was diluted with water and extracted with ether. The ether layer was washed twice with 5% sodium bicarbonate, twice with water and dried over sodium sulfate. On removal of the solvent, 22.45 g crude acetate was obtained. Application of heat and vacuum provided a pot residue of 19.5 g (91%) of the desired 11-dodecynyl acetate which was 99% pure as analyzed by GLC.

A suspension of $LiAlH_4$ (3.25 g, 86 mmol) in 500 ml anhydrous ether was stirred under nitrogen for 30 min in a 1 liter 3-necked flask equipped with a dropping funnel, reflux condenser, magnetic stirrer and heating mantle. To the flask was added dropwise the dodecynl acetate (19.5 g, 81 mmol) dissolved in ether. The reaction mixture was then stirred at room temperature for 30 min and at reflux for 45 min. Moist ether was added to the cooled solution to decompose the excess $LiAlH_4$. This was followed by the addition of a small amount of water. The solution was transferred to a separatory funnel and shaken with 300 ml 6 N H_2SO_4. The ether layer was washed with water, 5% $NaHCO_3$ solution, water and dried over sodium sulfate giving 13.65 g (75 mmol) 11-dodecynol. After the addition of 2.35 g crude alcohol from a previous run, the alcohol was reacted with dihydropyran to form the tetrapyranyl ether derivative, which was purified by preparative TLC, petroleum ether/ether (80:20, v/v), to give 14.3 g (61%) of the protected derivative of 11-dodecynol.

Preparation of a Halo Alcohol from a Dicarboxylic Acid (Fig. 2)

Preparation of 14-bromotetradecanol. 19.5 g (75.6 mmol) 1,12-dodecanedicarboxylic acid was converted to 21.5 (74.8 mmol) of the dimethyl ester by refluxing the acid in methanol containing 3% sulfuric acid. Reduction by 5.5 g (145 mmol) $LiAlH_4$ gave 14 g (60 mmol) of 1,14-tetradecanediol. The diol was placed in a 500-ml 3-necked flask equipped with a heating mantle, reflux condenser, thermometer and magnetic stirrer. To the flask was added about 120 ml 48% hydrobromic acid followed by the addition of 250 ml petroleum ether (bp 60-90 C). The mixture was then stirred and heated for several hours at 55-60 C. The petroleum ether phase was decanted and shaken with granular potassium carbonate. To the lower phase, 250 ml fresh petroleum ether was added, and the mixture was heated overnight. The phases were again separated, and the petroleum ether phase was shaken with granular potassium carbonate. The extracts were combined after being decanted through a filter, and the solvent was removed under vacuum to yield 10.45 g (58%) of the crude bromoalcohol. TLC analysis, petroleum ether/ether (60:40, v/v), indicated contamination by ca. 3% of dibromide and $<1\%$ of diol.

Preparation of *trans*-Alcohols (Fig. 3)

Lithium-ammonia Reduction of Heptadecynols: Trans-*11-heptadecenol.* A borosilicate glass cylinder measuring 57 mm x 12.5 cm was fabricated to fit a pressure vessel described below. To 20 ml freshly distilled tetrahydrofuran in the glass cylinder equipped with a glass enclosed magnetic stirring bar was added 500 mg (2 mmol) 11-heptadecynol. The glass cylinder was fitted with a dry-ice condenser equipped with a side arm, and ca. 60 ml of anhydrous

liquid ammonia was added by condensation. Gradually, with stirring, 500 mg lithium metal was added in small pieces. The intense blue color characteristic of the solvated electron persisted. The cylinder was then sealed in a steel pressure vessel equipped with a threaded cap and a needle valve for pressure release. The vessel was then placed in a steam bath and heated with stirring overnight. The next morning the pressure vessel was cooled in an ice bath, and the ammonia gas was gradually released by opening the valve in the cap. To the contents of the cylinder was added slowly moist ether to react with residual lithium metal. The mixture was extracted with additional ethyl ether. The organic layer was washed with 10% HCl, water and then dried over magnesium sulfate. GLC analysis of the reduced alcohol as the acetate indicated that 86% *trans* alcohol had been obtained with the acetylenic alcohol remaining as the impurity.

The *trans* alcohol (450 mg) was purified on a column (2.5 × 25 cm) containing 20 g acid-treated Florisil (60/100 mesh) containing 20% silver nitrate by weight. Elution of the *trans* alcohol was carried out with petroleum ether containing 5% diethyl ether in fractions of 75 ml. Those fractions containing the olefinic alcohol on the basis of the GLC analysis of the acetate were combined. The desired material was essentially eluted with a volume of 600 ml. After removal of the solvent, 280 mg (62%) *trans*-11-heptadecenol was obtained.

Preparation of *cis*-Alcohols (Fig. 3)

Semi-hydrogenation of Heptadecynols: Cis-*4-Heptadecenol.* In a 3-necked 100 ml flask equipped with a rubber septum, a high speed magnetic stirrer, a 2-way stopcock to admit either nitrogen or hydrogen and an outlet tube to a bubbler, was placed 20 ml of a 5 mmol solution of $Ni(OCOCH_3)_2 .4H_2O$ in 95% ethanol. The solution was stirred rapidly for a few minutes under nitrogen, the nitrogen flow discontinued, and hydrogen was added. To the solution was added 100 μl of 1.0 M $NaBH_4$ in 95% ethanol. After stirring for 20 min, 20 μl ethylene diamine was added, followed by a solution of 532 mg (2 mmol) 4-heptadecynol in 6 ml 95% ethanol. The mixture was stirred under a hydrogen flow for 1 hr. The contents were filtered through a small Darco-Celite column and washed with 95% ethanol. The eluant was diluted with water and extracted with petroleum ether. The organic layer was washed with water and dried over sodium sulfate. The solvent was evaporated, leaving 440 mg (87.2%) of a crystalline material. The IR spectrum indicated the absence of *trans* double bonds, and GLC analysis of the acetate showed it to be 99% pure *cis*-4-heptadecenol.

Chain Extension of *trans*-Heptadecenyl Substrates (Fig. 3)

Preparation of trans-*[1-^{14}C]octadecenoic Acids.* Approximately 200-300 mg of *trans*-heptadecenol was dissolved in 1 ml pyridine. The solution was cooled to 0 C, 500 μl methanesulfonyl chloride was added and the solution was kept at 0 C for 30 min and 1 hr at room temperature. The reaction mixture was then cooled to 0 C, and 25 ml of cold 10% hydrochloric acid was added. The mesylate was extracted with ether, and the ether layer was washed with 10% hydrochloric acid, then with saturated sodium bicarbonate solution and dried over Na_2SO_4. The crude mesylates were purified by TLC using benzene as the developing solvent. [Note that benzene is a suspected carcinogen and should be used with caution.]

Cyanation reactions to produce the labeled nitrile were conducted in small vials equipped with Teflon-lined caps containing 1.81 mg (27 μmol) of potassium cyanide (3.125 mCi). Approximately 8.3 mg (25 μmol) of the mesylate of the *trans* alcohols was added in ether solution. The ether was evaporated under nitrogen and 110 μl dimethyl sulfoxide (DMSO) was added. The vials were flushed under nitrogen, capped tightly and heated for 1 hr at 80-85 C. The vials were cooled, 0.5 ml water added and the contents extracted 4 times with 1 ml ether. The combined extracts were washed twice with dilute sodium bicarbonate and filtered through a small disposable pipette containing silica gel. The ether was concentrated to a small volume and the crude nitrile was applied to a sheet of ITLC-SA™ paper (Gelman Instrument Co., Ann Arbor, Michigan) which was developed with petroleum ether/ether (90:10, v/v). The paper was then sprayed lightly with a 0.1% ethanolic solution of 2,7-dichlorofluorescein and observed under UV light. The band containing the labeled nitrile was cut into small pieces and placed in a small screw cap vial. The vials were cooled under a stream of argon to 0 C, and 125 μl 33% hydrochloric acid in methanol was added. The vials were allowed to stand at room temperature for 2 hr, after which 12.5 μl concentrated hydrochloric acid was added and the vials were allowed to stand at room temperature for 2 more hr. The vials were cooled to 0 C, water was added and the methyl esters were extracted with 4 ml ether. The ether solution was washed once with water and 3 times with 10% sodium bicarbonate solution and the ether evaporated under nitrogen. TLC and GLC analysis showed that good conversion (> 98%) had taken place.

To the methyl esters was added about 0.5 ml 4% potassium hydroxide in 90% ethanol. The vials were flushed with nitrogen and heated at 80 C for 45 min, cooled and the contents acidified with 10% hydrochloric acid, extracted with ether and the ether layer was washed with sodium chloride solution. The ether solution was concentrated under nitrogen, and the residue was purified by TLC on a sheet of ITLC paper, developed with petroleum ether/ether (80:20, v/v). A small strip (~3-4 mm wide) was cut from the center of the paper, sprayed with sulfuric acid-dichromate and charred. Using this charred strip as a guide, the fatty acid band on the uncharred paper was located and cut into small pieces, placed in a vial and eluted with ether several times. The ether was then evaporated, a small volume of petroleum ether was added and the solution was filtered through glass wool in a disposable pipette and diluted to a volume of 5 ml. An aliquot of 5 µl was taken for scintillation counting to determine the amount of radioactivity present, and the extracted TLC papers were counted to check the residual activity. The yields of the several [1-^{14}C]octadecenoic acid *trans* isomers are given in Table 1.

TABLE 1

Yields of 1-^{14}C-Octadecenoic Acid Isomers Synthesized

1-^{14}C-Octadecenoic acid isomer	Radioactivity, µCi (% yield)	
	cis	*trans*
Δ 3	–	64 (2.0)
4	311 (10.0)	155 (5.0)
5	255 (8.2)	352 (11.3)
6	493 (15.8)	140 (4.5)
7	523 (16.7)	162 (5.2)
8	307 (9.8)	261 (8.4)
9	263 (8.4)	173 (5.5)
10	426 (13.6)	234 (7.5)
11	46 (1.5)	169 (5.4)
12	437 (14.0)	135 (4.3)
13	370 (11.8)	57 (1.8)
14	618 (20.0)	19 (0.6)
15	223 (7.1)	29 (0.9)
16	336 (10.8)	45 (1.4)
17	230 (7.4)	

Chain Extension of *cis*-Heptadecenyl Substrates (Fig. 3)

Preparation of cis-*1-*^{14}*C-octadecenoic acids.* The synthesis of the *cis* acids involved two modifications of the procedures for the synthesis of *trans* isomers. We felt that the low yields encountered in labeling of the *trans*-substrates were attributable to the excessive handling of the K^{14}CN. Therefore, prepackaged 3.9 mg K^{14}CN (3.125 mCi, American Radiochemical Co.) in each screw cap vial was used for chain elongation. Mesylate (19 mg) in 250 µl DMSO was added to each vial. The contents were flushed with argon and heated for 2 hr at 88 C. TLC indicated good conversion to the nitrile. The vial was cooled in an ice bath; 1 ml water was added followed by 1 ml ether. The ether layer was removed to another vial and the aqueous layer was extracted 3 times additionally with ether. The ether extracts were washed twice with saturated sodium bicarbonate. The second modification to the synthesis involved the use of alkaline hydrolysis, the nitrile to the fatty acid, since acidic hydrolysis of *cis*-nitriles had previously produced *cis*-fatty acids contaminated with undesired positional and geometric isomers (Janke, J., and Holman, R.T., unpublished results). After the ether was evaporated under nitrogen, 150 µl of 3.5 N ethanolic sodium hydroxide was added. The vial was heated overnight in a steam bath. To the cooled vial was added 1 ml water, and the contents were extracted several times with petroleum ether. The aqueous layer was acidified with HCl, and the fatty acids were extracted 3 times with ether and washed twice with saturated bicarbonate. TLC check of both extracts on ITLC paper (petroleum ether/ether/acetic acid [95:5:1, v/v/v]) showed the presence of unhydrolyzed nitrile in the petroleum ether. This necessitated the combination of the 2 extracts which were then hydrolyzed overnight with 1 ml of 8.75 N sodium hydroxide in 50% ethanol. The tubes were cooled, diluted with water and acidified with 20 drops of HCl. Then they were extracted 3 times with ether, and the ether extract was washed with sodium bicarbonate. The fatty acids were purified on individual sheets of ITLC paper using the same procedure described above for *trans* acids. The isomeric acids prepared and their total radioactivities are listed in Table 1.

Preparation of Octadecynoic Acids by Two Schemes

Coupling of an Alkyl Halide (Fig. 4). 4-Octadecynoic Acid (IV). Compound IV was prepared in a manner similar to compound II, using 10.0 g (59.5 mmol) of 2-(4-pentynyloxy)-tetrahydropyran in 60 ml THF, 38.0 ml (60.8 mmol) 1.6 M n-butyl lithium in hexane, and 15 g (57 mmol) 1-bromotridecane in 90 ml HMPA. After reacting for 1 hr at room temperature, workup gave 18 g (51 mmol) of crude protected 4-octadecynol. Removal of the protecting group followed by recrystallization from petroleum ether gave 10.8 g (71%) of 4-octadecynol as white crystals

melting at 40-41.5 C.

The alcohol (1.0 g) was oxidized to the aldehyde and then the acid as previously described (16) to yield 730 mg (70%) of IV as white crystals melting at 73-74.5 C (lit. 74-75 C [10]).

Coupling of a Terminal Alkyne (Fig. 5). 12-Octadecynoic Acid (V). Compound V was prepared in a manner similar to compound II by reacting 1.4 g (24 mmol) of 1-heptyne in 25 ml THF, 15 ml (24 mmol) 1.6 M n-butyl lithium in hexane, and 5.5 g (16 mmol) of 2-(11-bromoundecyloxy)-tetrahydropyran in 35 ml HMPA for 1 hr. Removal of the protecting group gave 4.05 g (95%) of crude 12-octadecynol. Two recrystallizations from petroleum ether yielded white crystals melting at 29.5-31 C (lit. 28-29 C [28]). TLC analysis using petroleum ether/ether (60:40, v/v) indicated one component.

The alcohol (2.0 g) was oxidized to yield 1.4 g (67%) 12-octadecynoic acid. One recrystallization from petroleum ether gave white crystals of V melting at 46-47 C (lit. 46-47 C [10]) with no impurities as judged by TLC using petroleum ether/ether/acetic acid (70:30:1, v/v/v).

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Hydrolysis of 4-Methylumbelliferyl Butyrate: A Convenient and Sensitive Fluorescent Assay for Lipase Activity

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ABSTRACT

A rapid, sensitive and convenient fluorescent assay was developed to screen for lipase activity. The non-fluorescent substrate, 4-methylumbelliferyl butyrate (4-MUB), solubilized in either liposomal dispersions or bile salt/lecithin mixed micelles, is hydrolyzed to butyric acid and the highly fluorescent compound, 4-methylumbelliferone (4-MU). Assays are run at 37 C for 10 min, terminated, and the changes in fluorescence quantitated with a Turner III fluorometer. Both lingual and pancreatic lipases exhibit activity against this artificial substrate. The assay has several advantages: nmoles 4 MU/ml/hr are measured allowing the detection of very low lipolytic activity; multiple samples may be simultaneously assayed, and only a brief incubation period is required.
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INTRODUCTION

Triglyceride lipases [EC 3.1.1.3] are enzymes that are essential for normal fat digestion; protein fractions containing lipolytic activity eluted from chromatography columns during lipase purification may be located by a variety of methods. Many conventional assays for lipolytic activity require expensive automated titration equipment (1,2) and utilize a stable triglyceride emulsion as physiological substrate (3,4). Although radioisotopic assays are very sensitive and specific (1), scintillation counting is required. Recent work on rat lingual lipase, including studies from this laboratory (5), has utilized small quantities of tissue as a purification source. It was, therefore, desirable to develop a rapid, convenient assay for the detection of minute quantities of lipase. Artificial fluorogenic substrates are enjoying increasing popularity for the assay of several enzymes including proteases (6), glycosidases (7) and phosphatases (8). This paper describes a simple, rapid and sensitive method for the qualitative assay of lipase activity employing fatty acid esters of 4-Methylumbelliferone (7-Hydroxy-4-Methylcoumarin).

PRINCIPLE OF THE METHOD

Several digestive lipases catalyze the hydrolysis of the fatty acid ester bond of the non-fluorescent compound, 4-MUB, to yield one molecule of the highly fluorescent compound, 4-MU, and one molecule of butyric acid (Fig. 1). Each molecule of 4-MU produced, therefore, reflects the hydrolysis of one molecule of fatty acid by the lipase.

MATERIALS AND METHODS

Crystalline 4-methylumbelliferyl butyrate, 4-methylumbelliferyl palmitate (4-MUP), PMSF (phenylmethylsulfonyl fluoride), Rhodamine 6G, and Triton X-100 were products of Sigma Chemical Company, St. Louis, Missouri. Tributyrin (glyceryl tributyrate) was purchased from Aldrich Chemical Co., Milwaukee, Wisconsin. Egg lecithin, grade 1, was obtained from Lipid Products, Redhill, Surrey, U.K., and was >99% pure by thin-layer chromatography using a solution of chloroform/methanol/acetic acid/water (25:5:4:2, v/v/v/v). Sodium Taurocholate was purchased from CalBiochem-Behring, LaJolla, California, and was >98% pure by thin-layer chromatography (isoamyl acetate/propionic acid/n-propanol/water, 4:3:2:1, v/v/v/v). Buffers (0.01 M glycine/HCl, pH 1-3, 0.01 M acetate pH4, 0.01 M citrate/NaH_2PO_4 [pH 5-7] and 0.01 and 1 M Tris/HCl [pH 7.5-10]) were made from ACS grade chemicals (Fisher Scientific Co., Pittsburgh, Pennsylvania). Millipore filters (pore size .22 μm) were purchased from Millipore Corporation, Bedford, Massachusetts. Methylcellusolve (ethylene glycol monomethyl ether) was obtained from Pierce Chemical Company, Rockford, Illinois. Sodium chloride (NaCl) was roasted at 600 C in a muffle furnace to oxidize impurities and water was ion-exchanged, filtered and double-distilled. All glassware was acid-alkali washed and oven dried. Cholesterol esterase [EC 3.1.1.13] was obtained from Boehringer Mannheim, Indianapolis, Indiana. Porcine type VI pancreatic lipase, porcine type IX trypsin and porcine pancreatic phospholipase A_2 were obtained from Sigma Chemical Company and dissolved in 150 mM NaCl prior to use. Rat lingual lipase was prepared as previously described (5). Colipase was purified by the method of Gaskin et al. (9). The presence of micelles was established by the spectral shift of Rhodamine 6G with a Cary-Varian 210

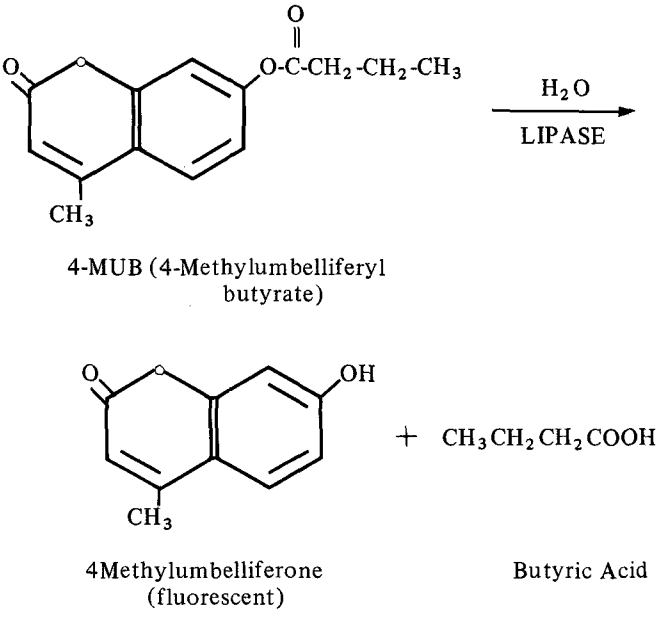

FIG. 1. Hydrolysis reaction on which the fluorescent lipase assay is based.

recording spectrophotometer as described by Carey and Small (10).

Assay Procedure

Assays were performed in 12 x 75 mm RTU tubes (Becton-Dickinson, Rutherford, New Jersey) with 0-20 μl of lipase (~20 μg protein), 20 μl of the appropriate pH buffer (see Materials and Methods) and 60 μl of substrate (see Substrates). For each assay, lipase reactions were conducted simultaneously at both 4 C in an ice-bath and 37 C for 10 min in a Dubnoff shaking incubator (Precision Scientific Co., Chicago, Illinois). All assays were performed in duplicate or triplicate. Reactions were quenched with 3 ml cold (4 C) 1 M Tris buffer, pH 7.5, and change in fluorescence quantitated with a Turner III fluorometer (Sequoia-Turner Co., Mountain View, California) utilizing excitation and emission settings of 365 nm and 450 nm respectively. The change in fluorescence was obtained by subtracting the reading of the 4 C incubated samples against the 37 C control samples for each assay. Fluorometer constants were calculated from standard stock solutions of 4-MU (8, 11). Heat-inactivated lipase (denatured at 70 C for 20 min) and "blank" (20 μl of 150 mM NaCl instead of lipase) at all pH buffers were run at 4 C and 37 C to establish "background" fluorescence values. The mean "background" values were subtracted from the lipase-catalyzed hydrolysis assays to exclude any changes in fluorescence due to non-enzymatic hydrolysis of 4-MUB.

"Background" values were negligible except at pH >8 (see Results). Lipase activity also was measured using tributyrin as substrate by pH STAT titration as previously described (5).

Substrates

[A] Suspensions of 4-MUB (2mM-10mM) were prepared by adding crystalline 4-MUB to 150 mM NaCl. The mixtures were vortex-mixed, followed by sonication with three 10-watt/sec bursts in a Branson S-75 sonicator (Branson Ultrasonics, Inc., Danbury, Connecticut). These fluorescent substrates were >99% excluded by a .22 μm Millipore filter indicating the limited solubility of 4-MUB in 150 mM NaCl alone. (Assays were run on substrates pre- and post-filtration to determine the degree to which 4-MUB remained solubilized after passing through the filter.)

[B] Liposomal dispersions of 2mM 4-MUB and 2mM egg lecithin were prepared using solubilization in chloroform/methanol (2:1, v/v). After evaporation under a N_2 stream and dessication at 23 C for 1-2 hr, the substrate was solubilized in 150 mM NaCl, vortex-mixed and sonicated as in [A] (Liposomal dispersions of 4-MUB were excluded >90% by a .22 μm Millipore filter). This substrate was used without

further filtration.

[C] Mixed micelles of .5 mM 4-MUB, 5 mM egg lecithin and 10 mM sodium taurocholate were prepared as in [A] except sonication was not performed. Mixtures were allowed to equilibrate for 24-72 hr prior to use. These preparations were filtered through .22 μm Millipore filters to remove unsolubilized 4-MUB. The concentration of 4-MUB solubilized in mixed micelles which passed through the filter was approximately 11 μM as judged by comparison to the baseline fluorescence of stock solutions of 4-MU (7,8). After filtration the solutions were optically clear; the presence of micelles was confirmed by the spectral shift of 2.5 μM Rhodamine 6G (10).

[D] Substrates with 4-MUP instead of 4-MUB were prepared as in [A] and [B].

RESULTS

Substrate Selection

Bile-salt lecithin mixed micellar and egg-lecithin liposomal dispersions were used for the solubilization of 4-MUB, as we were unable to obtain appreciable hydrolysis of the substrate by lipases when solubilization with 1-6% (v/v) ethylene glycol monomethyl ether (methylcellusolve), 1% (v/v) Triton X-100, 40% (v/v) methanol, 30% (v/v) ethanol or 10% (w/v) gum arabic was employed. Experiments with 4-MUB suspensions of different concentrations (2mM through 10 mM concentrations of 4-MUB, substrate A) also were performed; the coefficient of variation was unacceptably high (>30%) due to an inability to consistently solubilize the substrate.

Sensitivity and Reproducibility

The assay was able to detect lipolytic activity as low as 10 nanomole/ml/hr; this was 6000-fold more sensitive than conventional titration which measures μ moles/ml/min (2). The assay was linear with respect to protein concentration and substrate concentration for up to 15 min of incubation. The coefficient of-variation for the assay was 13.5%, when the egg-lecithin liposomal (n=10) and 5.8% (n=10), when the bile-salt lecithin mixed micellar dispersion was used. The initial experiments indicated that the hydrolysis of 4-MUB by lingual and pancreatic lipases was approximately 8-10 times that of 4-MUP solubilized in the same manner to 4-MUB (see Substrates). Therefore, 4-MUB was chosen to be used for the subsequent studies. Lipase activity as measured by the fluorescent assay was less than .03% of the activity obtained by pH STAT titration.

Specificity

Negligible activity on 4-MUB was detected with either phospholipase A_2 or trypsin, and lipolytic activity was not inhibited by .5-1 mM PMSF. We were able to detect hydrolysis of the substrates by cholesterol esterase at pH7. However, hydrolysis of 4-MUP was 15 times greater than the hydrolysis of 4-MUB (212 ± 10 nmoles/ml/hr vs. 3601 ± 800 nmoles/ml/hr). At pH 4 hydrolysis of both substrates by cholesterol esterase was less than 1/6 of the activity at pH 7-8. At pH 8-10, spontaneous alkaline hydrolysis of 4-MUB to 4-MU produced background fluorescence in the "blank" controls which was 5-10 times that of controls at pH 7 or lower. This considerable background must be subtracted from lipase-catalyzed assays at these extremely alkaline pH's. Figure 2 depicts 4-MUB hydrolysis as a function of pH for rat lingual lipase. A pH optimum of 4-5 on the artificial substrate was seen in agreement with previous studies on triglyceride (physiological) substrates (5,12,13). Pancreatic lipase demonstrated optimal activity on 4-MUB at pH 7 (392 ± 20 nmoles/ml/hr) which was approximately 20 times the activity at pH 1-2 (19 ± 4 nmoles/ml/hr). Exogenous colipase (15 units/ml) did not stimulate hydrolysis of either 4-MUB or 4-MUP, and hydrolysis of the substrates by pancreatic lipase was inhibited by 1% Triton X-100. Substrates were stable for up to 5 days when stored at 4 C.

DISCUSSION

There are several distinct advantages of the proposed assay over conventional methods for monitoring lipase activity. First, the fluorescent assay allows multiple samples to be run simultaneously; conventional pH STAT titration devices are able to perform only one assay at a time. Second, the assay is rapid, requiring only a 10-15 min incubation at 37 C. Third, the high sensitivity of the assay allows the detection of extremely low levels of lipolytic activity quite conveniently, unlike radioisotopic assays, which require extraction of the products of lipolysis and scintillation counting (1). The fact that both pancreatic and lingual lipases hydrolyze 4-MUB and 4-MUP in the presence of taurocholate and lecithin is an additional advantage of the assay. Taurocholate and lecithin inhibit the hydrolytic activity of pure pancreatic lipase when triglycerides, in contrast to 4-MUB, are used as substrates (5). Hydrolysis of the fluorescent substrates by pancreatic lipase was inhibited by Triton X-100, a non-ionic detergent, which also inhibits activity on triglycerides (14). Activity of pancreatic lipase on the fluorescent

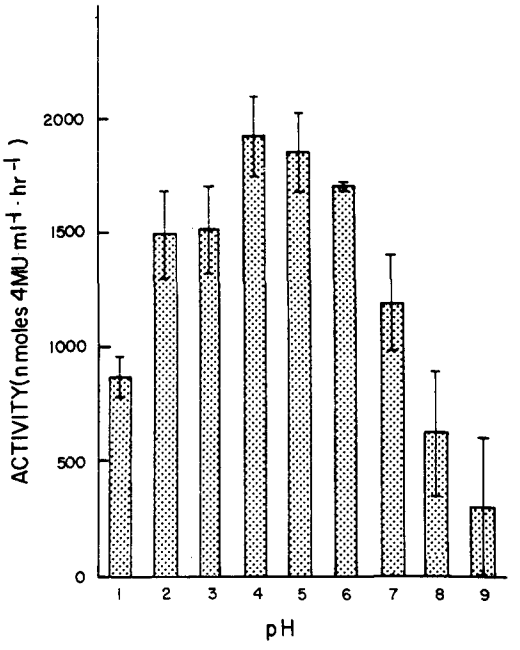

FIG. 2. pH-Dependence of lingual lipase activity (4-methylumbelliferyl butyrate liposomal dispersions). Hydrolytic activity was tested in the pH range from 1.0-9.0. The reaction conditions were: 37 C, total volume 100 μl, 60 μl substrate (see Substrates for method of preparation), 20 μl lingual lipase, 20 μl buffer (see Materials and Methods for buffers used). The data are expressed as means ± S.E.

substrates was not, however, enhanced by colipase. It is possible, therefore, that pancreatic lipase may be exhibiting esterase activity on the fluorescent compounds instead of specific triglyceride lipase activity (15).

Minimal hydrolysis of either 4-MUB or 4-MUP was observed with phospholipase A_2 or trypsin. PMSF, an esterase-serine protease inhibitor, did not affect hydrolysis of the substrates. Cholesterol esterase, however, was able to hydrolyze 4-MUP at a higher rate than 4-MUB. This higher rate of hydrolysis of 4-MUP by cholesterol esterase is of interest and may reflect differences in affinity for the two substrates (stereochemically, 4-MUP more closely resembles cholesterol palmitate, a preferred substrate for the enzyme [16], than does 4-MUB). Although the assay may be conducted at pH 1-9, it can be expected to be sensitive and specific for acid lipases (i.e. lingual and lysosomal lipases) (11) at pH 4, particularly if 4-MUB is used as the substrate.

While both fluorescent substrates–the one dispersed in liposomes and the other dispersed in mixed micelles–are hydrolyzed by lipases, the latter appears to be the substrate of choice due to its higher precision (c.v. 5.8%). Previous investigators have reported similar fluorescent assays for lipases, but have used methylcellusolve and other compounds to disperse the substrate (17-20). The present assay represents a significant improvement, since previous methods fail to solubilize the fluorescent substrate satisfactorily and are, therefore, of limited value in the detection of low levels of lipase activity for rapid screening purposes. Although lipase-catalyzed hydrolysis of the artificial fluorescent substrate dispersed in liposomes or mixed micelles is less than .03% of that of tributyrin, the assay is qualitatively quite sensitive. This is especially important as the fluorescent assay will uncover lipase activity missed by techniques such as the pH STAT which is 6,000-fold less sensitive. This increase in sensitivity is particularly useful when purifying a lipase from small quantities of tissue (i.e. rat lingual lipase). Brockerhoff also has observed that fluorescent esters and other water-soluble compounds were hydrolyzed at a less rapid rate (.3%) than the physiological long-chain triglyceride substrate, triolein (21). Lipases when adsorbed to the oil-water interface of an emulsion particle undergo "activation" leading to a rapid increase in enzyme activity in contrast to soluble esterases which do not exhibit this phenomenon (22). The fluorescent substrates, unlike physiological emulsions, are hydrolyzed at an extremely slow rate. This slow rate of hydrolysis of the artificial fluorescent esters may be due to a lack of interfacial "activation" on substrates solubilized in liposomes or mixed micelles, or may simply reflect the non-triglyceride nature of the substrates, and therefore, a degree of non-specific esterase activity. Although an artificial fluorescent substrate is utilized, and despite the possible limitations in specificity of the method, the ability to perform multiple assays simultaneously should be especially helpful in detecting column chromatographic fractions with lipolytic activity and in identifying lipolytic protein bands directly on or eluted from polyacrylamide gels. In conclusion, the ease of the performance, as well as the rapidity, sensitivity and reproducibility of the assay render it a particularly valuable tool in ascertaining low levels of lipase activity and in monitoring lipase purification procedures.

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COMMUNICATIONS

Pulmonary Lipid Peroxides and Fatty Acids of Rats Fed Different Lipids and Exposed to Oxygen at Hyperbaric Pressure

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ABSTRACT

Semipurified diets containing different lipids were fed to rat dams during lactation and subsequently to their pups for 33 weeks post-weaning. Some rats within each group were exposed to oxygen at hyperbaric pressure (OHP). Lipid peroxide levels were lower in lungs of rats fed 7% hydrogenated coconut oil or 10% butter as compared with their controls, fed 7% corn oil or 10% safflower oil, respectively. Exposure to OHP increased lung peroxide levels. This increase varied with the type of fat in the diet. Studies of the fatty acid composition indicate that lipid peroxide levels generally increased with an increase in the levels of 18:2 in lung total lipids. The results suggest that the type of dietary lipid may alter the susceptibility of the animal to pulmonary oxygen toxicity.
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INTRODUCTION

Oxygen at pressures greater than 1 atmosphere (hyperbaric oxygenation) is used in medicine for the treatment of different diseases such as hyaline membrane disease and atelectasis. One of the major concerns in the prolonged therapeutic administration of oxygen at hyperbaric pressure (OHP) has been that of pulmonary oxygen toxicity (1-4). Although the exact biochemical basis of pulmonary oxygen toxicity is quite complex, it has been equated in part with the formation of lipid peroxides (5, 6). Changes in fatty acid composition of some phospholipids of lung and lavage as a result of OHP treatment have been observed (7,8). There are no studies, however, on the effect of OHP on pulmonary lipid peroxides in animals fed different dietary lipids.

In one study (9), the effect of dietary fatty acids on lung lipids in relation to oxygen toxicity was investigated. Rats fed three different diets for 33 days were exposed to 100% oxygen. Higher mortality was observed in rats fed a saturated fat diet (10% hydrogenated coconut oil (HCO) + 2% standard rat chow) as compared with those fed 10% cod liver oil or the standard rat chow.

It is well established that the fatty acid composition of lungs, like that of other animal tissues, is affected by hormonal and nutritional factors. In a previous study (10) we observed that the feeding of diets containing different lipids during lactation and thereafter to their offspring resulted in modifications in the fatty acid composition of total lipids and phospholipids in rat lung and lavage. Two nutritional models, an essential fatty acid (EFA) deficiency and the feeding of saturated versus unsaturated fats, were used in this study. Using the same two nutritional models, we have tested the hypothesis that if the rats with different pulmonary fatty acid composition are exposed to OHP, the levels of lipid peroxides should vary depending upon the fatty acid composition of the lung.

MATERIALS AND METHODS

The experimental design of the feeding study was essentially similar to the one previously described (10). Semipurified diets (basal diet AIN-76) containing 7% corn oil (control group for EFA deficiency model) or 7% HCO (experimental), 10% safflower oil (control group for saturated versus unsaturated fats model) or 10% butter (experimental) were fed ad libitum to Sprague-Dawley rats. Diets were freshly prepared every 2-3 weeks and stored at 4 C. Butylated hydroxytoluene (BHT) was added as an antioxidant at 0.02% of the diet (11). The feeding of diets was initiated to the dams immediately after delivery of their pups and continued throughout 3 weeks of lactation. Pups were weaned, maintained in individual cages and fed the same diets as previously fed to their mothers. After a total of 36 weeks of feeding (3 weeks of lactation and 33 weeks post-weaning), rats within each dietary group were divided into two sub-groups. One group was exposed to OHP and the other served as control, unexposed to OHP.

For exposure to OHP, rats were placed in the hyperbaric oxygen chamber and the oxygen pressure was gradually (3-5 min) increased to 60 psi. This pressure was maintained for 20 min and was then gradually decreased to the atmo-

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spheric pressure over a period of 5 min. At the end of the treatment with OHP, rats (control as well as OHP exposed) were killed. The lungs were removed, chopped into small pieces and washed several times with cold physiological saline to remove any traces of blood. The lung tissue was homogenized in a Potter Elvejhem homogenizer with 9-10 volumes of physiological saline. Aliquots of the homogenates were used for the determination of total protein by biuret reaction (12) and lipid peroxides by the method of Ohkawa et al., using the thiobarbituric acid reaction (13). Aliquots of the lung homogenate were extracted for total lipids by using Bligh and Dyer's procedure (14). Portions of the total lipid extracts were used for the determination of their fatty acid composition using the procedures previously described (10).

The differences between the corn oil versus HCO groups (EFA deficiency model) and between the safflower oil versus groups fed butter (saturated versus unsaturated fats) were compared statistically using Student's t test. Similar comparisons were made between the groups exposed to OHP versus the groups without OHP using Student's t test.

RESULTS

The final body weights of rats fed an EFA-deficient diet (7% HCO) were significantly lower ($P < 0.001$) compared with their controls, 7% CO (384 7 ± 0.72 g vs 572.5 ± 10.54 g, mean ± SE). No significant diference was found in body weights of rats fed 10% butter compared with their controls, 10% safflower oil (578.2 ± 17.38 g for butter and 593.8 ± 25.5 g for safflower oil).

The effect of exposure to OHP on lipid peroxide content of lung in rats fed the various diets is shown in Table 1. The concentrations of lipid peroxides were significantly lower in lungs of rats fed diets containing 7% HCO or 10% butter as compared with their control groups, 7% corn oil or 10% safflower oil respectively. In all four dietary groups, the exposure of rats to OHP resulted in an increase in the peroxide levels in their lung. This increase was, however, significant only in rats fed 7% corn oil or 10% butter.

The results on fatty acid composition of lung total lipids in rats fed different diets with and without OHP treatment are shown in Table 2. The fatty acid composition of total lipids in lung was altered as a result of exposure to OHP. These modifications in fatty acid profiles, however, varied from one dietary treatment to another. In groups fed corn oil or safflower oil, a decrease in the proportions of 18:2 and corresponding increases in the proportions of 16:0 and 18:0 were generally observed. In total lipids of lungs of rats fed diets containing 7% HCO or 10% butter, the levels of 18:2 were much smaller as compared with their respective control groups. The exposure of EFA-deficient rats (7% HCO group) to OHP had essentially no significant effect on the fatty acid patterns of lung total lipids. In rats fed 10% butter, there was a significant increase in the proportions of 16:0 and 18:0 and a non-significant decrease in the levels of 16:1, 18:1, 22:3 and 22:4.

Changes characteristic of EFA deficiency were observed in the fatty acid composition of lung total lipids in rats fed 7% HCO as compared with the control group fed 7% corn oil. These changes consisted of increases in the proportions of 16:1, 18:1, 20:3ω9 and 22:3ω9 along with a decrease in the levels of 18:2, 20:4 and 22:4 in the EFA-deficient group. Similar changes of EFA deficiency in the fatty acid composition, except for 20:4 levels, also were observed in the pulmonary total lipids of rats fed 10% butter. The levels of arachidonic acid were essentially the same whether butter or safflower oil was the source of dietary fat.

DISCUSSION

Although oxygen is essential for the survival of animals, high levels of oxygen are considered to be toxic (15,16). Lung damage is one of the manifestations of oxygen toxicity. The biochemical basis for pulmonary toxicity due to OHP exposure is rather complex. In addition to the reduction in the levels of the surface active compound in the lung (7,8), several other factors also may be involved. One such mechanism would be an increase in peroxide levels in the lung. Indeed, pulmonary oxygen toxicity has been equated to some extent with lipid peroxide levels in the lung (5,6).

Tissue peroxide levels are controlled by several factors, including the type of fatty acids present in the tissue and its ability to dispose of lipid peroxides. The protective cellular mechanisms present in lung tissue which are affected by high oxygen tension include superoxide dismutase, catalase and glutathione peroxidase (17-19), along with vitamin E (20). Although we did not measure vitamin E levels in the lung, it has been observed that pulmonary vitamin E levels are rather unaffected even after feeding large amounts of vitamin E to rats (21) and mice (22).

Our working hypothesis was that the long term feeding of different dietary lipids initiated during early stages of life would result in maximizing the changes in fatty acid composition of

TABLE 1

Effect of Oxygen at Hyperbaric Pressure (OHP) on Lipid Peroxide Levels in Lungs of Rats Fed Diets Containing Different Lipids

Dietary lipids	Lipid peroxide levels		
	W/O OHP	OHP	Difference due to OHP
7% CO	2.24 ± 0.25	4.52 ± 0.34	$P < 0.001$
7% HCO	1.29 ± 0.11[a]	2.37 ± 0.47[b]	N.S.
10% Safflower oil	4.05 ± 0.35	4.86 ± 0.39	N.S.
10% Butter	1.23 ± 0.14[c]	1.93 ± 0.07[c]	$P < 0.01$

Values (mean ± SE) for lipid peroxides are nmol/malondialdehyde/mg protein, 4 rats/group in W/O OHP groups, 6 rats/group in OHP groups.

Values with a superscript in each column are significantly different from their corresponding control values ($^aP < 0.02$, $^bP < 0.01$, $^cP < 0.001$); N.S. = Not Significant.

CO = corn oil, HCO = hydrogenated coconut oil, W/O OHP = without OHP treatment.

lung lipids. Therefore, the response to OHP exposure of the animals raised on these diets would vary depending upon the type of diet fed and the fatty acid composition of the lung. Indeed, a higher peroxide content was found in lungs of rats fed corn oil or safflower oil compared with those fed HCO or butter. The highest levels of peroxide were observed in rats fed safflower oil, which supplied the highest levels of linoleic acid, about 75% of the total fatty acids. The peroxide levels in the lung were generally higher with an increase in its linoleic acid content. Although there was a general increase in the lipid peroxide levels as a result of OHP treatment, it was significant only in rats fed diets containing corn oil or butter. Due to the small levels of peroxides present in lung tissue of rats fed butter, the OHP-related increase in lipid peroxides may not be biologically significant. In view of the fact that the pulmonary linoleic acid levels were the highest in rats fed diets containing 10% safflower oil, it is rather perplexing that the exposure to OHP did not result in an increase in lung peroxide levels in this group. A possible explanation for this observation is that the peroxide content in lung already may have reached a "saturated" level so that exposure to OHP led to no additional increase. It also is conceivable that in this group the rate of peroxide breakdown increased along with the rate of their formation on OHP exposure so that very little additional accumulation of peroxides took place.

Our values for lipid peroxides in rat lung homogenate seem to be somewhat higher than those previously reported (13). This does not appear to be due to an artifact produced during the procedure of measurement used, because the addition of BHT to a rat lung homogenate had no effect on the lipid peroxide value (Alam, S.Q., unpublished data).

The levels of lipid peroxides after the OHP exposure were still lower in rats fed the EFA-deficient diet (7% HCO) or the one with saturated fat (10% butter) as compared with their control groups. If lipid peroxides do play a role in pulmonary oxygen toxicity, our observation suggests that the rats fed saturated fat such as butter or an EFA-deficient diet would be less susceptible to pulmonary oxygen toxicity.

The implication of our results that EFA deficiency or the feeding of saturated fat such as butter may lower the susceptibility to pulmonary oxygen toxicity is not consistent with the observations of Kehrer and Autor (9), who found higher mortality when rats fed a saturated fat, 10% HCO + 2% standard rat chow diet, were exposed to 100% oxygen. It was suggested by these investigators that an increase in the saturated fatty acid content of lung triglycerides through dietary manipulation resulted in increased susceptibility to oxygen toxicity. It may be pointed out, however, that in addition to the dietary fat, other variables such as the differences in the basal diets among the groups and the addition of 2% standard diet to the diet containing 10% HCO also were present. It is rather difficult to attribute the observed differences in susceptibility of the animals to the nature of the dietary fatty acid alone. Therefore, the question of the relationship between the diet induced changes in fatty acid composition of lung and susceptibility to OHP still remains unanswered and needs further investigation.

TABLE 2

Effect of Oxygen at Hyperbaric Pressure (OHP) on the Fatty Acid Composition of Total Lipids in Lungs of Rats Fed Diets Containing Different Lipids

Fatty acid	7% CO		7% HCO		10% Safflower oil		10% Butter	
	W/O OHP	OHP	W/O OHP	OHP	W/O OHP	OHP	W/O OHP	OHP
14:0	0.9 ± 0.17	0.9 ± 0.25	2.6 ± 0.40	1.7 ± 0.22	0.9 ± 0.11	0.8 ± 0.06	1.9 ± 0.14	1.7 ± 0.10
16:0	29.8 ± 0.98	36.9 ± 1.01†	31.1 ± 0.35	30.6 ± 0.58	27.9 ± 1.53	37.6 ± 2.22†	31.8 ± 0.35	36.7 ± 1.18†
16:1	6.2 ± 0.82	6.7 ± 0.54	9.5 ± 1.23*	11.8 ± 0.51*	4.0 ± 0.59	3.0 ± 0.5	9.8 ± 0.35*	8.3 ± 1.33*
18:0	6.8 ± 0.58	8.2 ± 0.31	6.9 ± 0.68	7.3 ± 0.51	6.2 ± 0.32	9.5 ± 0.66†	6.7 ± 0.47	8.3 ± 0.48†
18:1	23.0 ± 1.40	24.4 ± 0.82	35.6 ± 2.94*	34.8 ± 2.15*	14.4 ± 1.16	15.9 ± 0.79	33.4 ± 1.13*	32.3 ± 1.20*
18:2	20.1 ± 1.16	14.3 ± 1.08†	0.1 ± 0.04*	1.3 ± 0.67*	29.6 ± 1.65	22.1 ± 2.56†	1.7 ± 0.32*	2.8 ± 1.42*
20:3ω9	0.6 ± 0.08	–	6.8 ± 1.30*	7.3 ± 1.28*	1.0 ± 0.19	–	1.6 ± 0.14*	1.4 ± 0.16*
20:4ω6	7.0 ± 0.79	6.6 ± 0.20	1.1 ± 0.21*	2.5 ± 0.35*	6.2 ± 0.84	7.6 ± 0.81	6.0 ± 0.57	7.4 ± 0.28
22:3ω9	0.6 ± 0.06	–	2.6 ± 0.51*	2.0 ± 0.31*	0.6 ± 0.06	–	1.3 ± 0.09*	0.6 ± 0.09*
22:4ω6	2.4 ± 0.26	1.4 ± 0.08	0.8 ± 0.15*	–*	2.2 ± 0.25	2.6 ± 0.56	1.8 ± 0.21	0.4 ± 0.20*

†Significantly different from controls, W/O OHP treatment, $P < 0.05$.

*Change resulting from diet (HCO versus CO and Butter versus Safflower oil), $P < 0.05$.

Values are area percent (mean ± SE); 4 rats/group in W/O OHP groups, 6 rats/group in OHP groups.

CO = corn oil, HCO = hydrogenated coconut oil, W/O OHP = without OHP treatment.

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Increases in Serum Sphingomyelin by 17β-Estradiol

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ABSTRACT

The effects of estrogens on plasma sphingomyelin and the hepatic activity of the initial enzyme of sphingomyelin synthesis were examined using immature chicks. After three days of 17β-estradiol administration, serum sphingomyelin, total phospholipids, and cholesterol doubled, and triacylglycerol levels increased 7.5 fold. The sphingomyelin content and percentage of total phospholipids of liver were unaffected by estrogen treatment. The specific activity of serine palmitoyltransferase (EC 2.3.1.50) was unchanged, but the total activity appeared slightly higher due to increased liver weights. The higher spingomyelin may, therefore, be due less to increased levels of biosynthetic enzymes than to factors such as the substrate (i.e., fatty acid) supply or decreased clearance of plasma sphingomyelin. These results are similar to earlier findings with key enzymes of cholesterol and glycerolipid biosynthesis and suggest that the three lipid pathways may be coordinated during estrogen treatment and enhanced very-low density lipoprotein (VLDL) synthesis.
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INTRODUCTION

Sphingolipids are prominent components of cellular membranes, lipoproteins and other structures, such as atherosclerotic plaques (1-3). They are composed of a long-chain or sphingoid base (sphingosine, sphinganine, phytosphingosine and their homologs), an amide-linked fatty acid and a polar headgroup (i.e., phosphorylcholine for sphingomyelin and carbohydrates for glycolipids). Sphingolipid (especially long-chain base) formation is one of the least understood aspects of the complex biosynthetic and sorting processes that yield lipids for hepatic membranes, lipoproteins and bile (4). We have been studying the initial enzyme of long-chain base biosynthesis (serine palmitoyltransferase, EC 2.3.1.50) to elucidate its properties and evaluate its role in regulating sphingolipid formation by liver (5-7). Comparisons of different tissues have revealed a clear relationship between serine palmitoyltransferase activities and the sphingomyelin composition of these tissues (6,7).

A useful model for additional studies of long-chain base metabolism is the estrogen-treated chick, which exhibits greatly enhanced fatty acid (8), glycerolipid (8-10) and cholesterol (11) biosynthesis and very-low-density lipoprotein (VLDL) formation and secretion (12). Since sphingomyelin is a constituent of VLDL and increased incorporation of radiolabeled fatty acids into sphingomyelin has been observed (13), this study examined chicken plasma to establish whether or not sphingomyelin levels were elevated. Finding this to be the case, the hepatic activity of serine palmitoyltransferase was measured to determine if this involves changes in this enzymatic activity.

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MATERIALS AND METHODS

Immature (10 to 15 days old) broiler chickens were injected for 3 days with either 17β-estradiol (Sigma) (20 mg/kg body weight/day) in propylene glycol or an equivalent volume of propylene glycol (12). They were killed by decapitation, blood was collected, and the livers were removed, weighed and placed on ice. The blood was left to clot at room temperature for 1 hr, centrifuged at 18,000 × g for 15 min, and the supernatants were stored in aliquots at −80 C. The livers were used to prepare a crude homogenate fraction and microsomes as described by Williams et al. (5), which were stored at −80 C until assay (no loss of activity was found when fresh and frozen samples were compared).

Lipid Analyses

Serum cholesterol and triacylglycerols were quantitated using an automated analyzer and COBAS reagents (Roche Analytical Instruments, Inc.) based upon the methods of Allain et al. (14) and Printer et al. (15), respectively. Phospholipids were determined by extracting 0.5 ml aliquots of serum or 0.5 g of liver according to Bligh and Dyer (16) and measuring the phosphate content (17). Sphingomyelin was analyzed by separating the phospholipids using two-dimensional thin layer chromatography and quantitating the phosphate content of the

sphingomyelin region of the plates (18). The recovery of the sphingomyelin was greater than 90%, based upon the results with samples spiked with known amounts of this lipid.

Enzyme Assays

Serine palmitoyltransferase was assayed by following the incorporation of [^{3}H]serine into 3-ketosphinganine as described in Williams et al. (5). The rates were established to be linear over the times used and proportional to the amount of protein added. Protein was assayed by the method of Bensadoun and Weinstein (19).

RESULTS AND DISCUSSION

Shown in Table 1 are the serum levels of cholesterol, triacylglycerols, total phospholipids and sphingomyelin for control and estrogenized chicks. As has been reported by others, estrogen treatment induced hyperlipemia with 2-fold higher cholesterol levels (11) and considerably greater triacylglycerol concentrations (9). Accompanying these changes were 2-fold elevations in total phospholipids and sphingomyelin (Table 1). The ratios of cholesterol:total phospholipids:sphingomyelin remained constant, and only the triacylglycerols increased disproportionately. 17β-Estradiol did not alter the sphingomyelin levels or proportions in liver. Control and estrogen-treated chicks had, respectively, 0.41 ± 0.09 and 0.39 ± 0.10 μmoles sphingomyelin/g liver (n = 6 of each), which represented 3.9 ± 0.5% and 4.4 ± 0.8% of the total phospholipids for these samples.

Since increases in long-chain base and sphingomyelin synthesis might be related to changes in the initial enzyme of this pathway (serine palmitoyltransferase), the total and specific activities of liver microsomes were

TABLE 1

Comparison of Serum Lipids of Chicks With or Without 17β-estradiol Treatment[a]

Property	Control chicks		Estrogen-treated chicks		P
	Amount	Ratio[b]	Amount	Ratio[b]	
Cholesterol (mg/100 ml)	128 ± 4.4	21.7	222 ± 46	19.7	<0.01
Triacylglycerol (mg/100 ml)	151 ± 74	13.7	1137 ± 454	54.1	<0.05
Phospholipids (μmoles/ml)	1.54 ± 0.27	9.8	2.9 ± 0.27	9.7	<0.05
Sphingomyelin (nmoles/ml)	157 ± 55	1.0	302 ± 92	1.0	<0.01

[a]The data represent mean ± S.D. for three to six animals. The significance of the differences was evaluated using the Student's t-test.

[b]The molar ratios were calculated using an estimated molecular weight of 700 for the triacylglycerols.

TABLE 2

Comparison of Liver Weights, Microsomal Protein and Serine Palmitoyltransferase Activities of Chicks With and Without 17β-estradiol Treatment[a]

Property	Control chicks	Estrogen-treated chicks	P
Liver weight (g/100 g B.W.)	3.8 ± 0.3	5.5 ± 0.1	<0.01
Microsomal protein (mg/g liver)	19.5 ± 2.9	20.3 ± 3.9	NS
Serine palmitoyltransferase			
Total activity (nmoles/min/100 g B.W.)	8.9 ± 2.5	12.7 ± 2.1	<0.1
Specific activity (pmoles/min/mg microsomal protein)	112 ± 6.4	119 ± 7.2	NS

[a]The data represent mean ± S.D. for triplicate assays of three to six animals. The significance of the differences was evaluated using the Student's t-test.

measured (Table 2). No change in specific activity was found (in addition, adult laying hens had similar activities, 95 ± 17 pmoles/min/mg); the total activity appeared slightly higher (43%) due to an increase in liver weight and total microsomal protein. The microsomal activities were an accurate representation of the total cellular enzyme, because the same percentage of the total activity (39.9 ± 9.0%) was recovered in the microsomal fraction for control and estrogen-treated chicks. Coleman et al. (12) have noted that the recovery of microsomal enzymes is generally poorer with immature chickens.

The serine palmitoyltransferase activity of chicken liver (2.3 nmol/min/g of liver, calculated from Table 2 with correction for the 40% recovery of total activity in the microsomal fraction), was approximately 3.8% of the glycerol 3-phosphate acyltransferase activity (63 nmol/min/g, calculated from data in ref. 9). This percentage was remarkably similar to the relative amounts of sphingomyelin compared to the total phospholipids of chicken liver (4.2%) or the total glycerolipids in serum from control chicks (4.3%). Hence, it is possible that the ratio of these activities is optimal for the partitioning of a common fatty acyl-CoA pool between sphingolipid and glycerolipid biosynthesis.

This investigation demonstrated that 17β-estradiol increases plasma sphingomyelin as predicted from its elevation of lipoprotein and sphingomyelin formation and excretion (13, 20). Since the activity of serine palmitoyltransferase was relatively unchanged, either long-chain base synthesis is not limiting or estrogens may act indirectly, for example, by increasing fatty acid synthesis (8) or decreasing the clearance of plasma lipoproteins. Interestingly, these results are analogous to findings with the key enzymes of glycerolipid and cholesterol biosynthesis. Luskey et al. (11) reported that the specific activity of β-hydroxy β-methyl glutaryl-CoA (HMG-CoA) reductase from estrogenized chicks was unchanged, despite higher plasma levels of cholesterol and increased hepatic synthesis. Coleman et al. (12) also found that, while estrogen treatment (with diethylstilbestrol) increased the liver weight and total activities of glycerolipid-synthesizing enzymes, the specific activities were not elevated. This may indicate that the relative activities of the initial enzymes of glycerolipid, sphingolipid and cholesterol biosynthesis are already balanced to yield the correct proportions of these lipids, which did not change except for triacylglycerols. Hence, greater sphingomyelin percentages may be achieved by changing the specific activity of serine palmitoyltransferase (6,7), whereas greater amounts with no change in phospholipid ratios (as occurs in VLDL synthesis) may be achieved by increasing the substrate supply and levels of many microsomal proteins. The similarities among most of the components of these three different lipid classes might indicate their coordinate regulation by estrogens.

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Adipocyte Fatty Acid Mobilization in vivo: Effects of Age and Anatomical Location

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ABSTRACT

The objectives of the present study were to determine if adipocyte triglyceride fatty acid (TGFA) mobilization in vivo varied among the different adipose tissue depots and whether these rates were affected by age. In order to accomplish these objectives, two groups of rats were studied. The first group initially weighed 84 ± 1 and the second group 333 ± 2. Both groups were placed on a semi-synthetic diet containing 6% corn oil (w/w) and 14% triundecanoin (w/w) for a period of 4 wk. Triundecanoin contains an 11-carbon (C-11) fatty acid (undecanoic acid) that was used to label the adipocyte TGFA. At the end of the 4-wk feeding period, triundecanoin was removed from the diet and replaced with an equivalent amount of corn oil. At this time and at weekly intervals for the next 4 wk, 5 rats from each age group were killed for the determination of TGFA composition in isolated adipocytes from the epididymal (Epi), perirenal (PR), subcutaneous (SC) and mesenteric (M) adipose tissue depots. When the content of C-11 was expressed as mole percent of the total fatty acids, mobilization was significantly more rapid from the PR and M depots than in the other two depots in the young rats. In the older rats mobilization was significantly slower in all depots compared to the younger group. The rates of mobilization were not different between the depots in the older animals. Since fat cell size continued to increase throughout the duration of the study, part of the decrease in C-11 content can be accounted for by dilution by newly acquired TGFA. When data were compared on the basis of the actual pmoles of C-11 per cell, rates of mobilization were not different between the depots nor was mobilization affected by age. These results emphasize again the impact the manner in which adipocyte metabolic data are expressed has on interpretation(s) when comparing adipocytes of different depots or from rats of different age.

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INTRODUCTION

Many estimates have been made of triglyceride fatty acid (TGFA) turnover in adipose tissue of man and experimental animals (1). These estimates vary widely and may be influenced by a diffuse set of variables which include species, age, sex, degree of adipocity and diet. The observed rate of turnover is also influenced by the extent to which mobilized fatty acids recycle back to adipose tissue by way of very low density lipoproteins (VLDL) (1).

Numerous in vitro studies have examined the rates of both basal and hormone-stimulated lipolysis in isolated adipocytes. Several of these studies have reported elevated basal lipolysis (based on glycerol release) in large adipocytes (2-6), whereas catecholamine-stimulated lipolysis was either unchanged (2-5,7,8), elevated (6, 9-12), or decreased in large adipocytes (13,14). In a previous study, this investigator observed an age dependent decrease in the lipolytic response of rat adipocytes to norepinephrine in three of four adipose tissue depots examined (4,5). The only exception was the epididymal depot in which no change in responsiveness to the catecholamine was found. Likewise, basal lipolysis did not differ among the depots nor was it affected by differences in age or cell size.

Despite the fact that isolated cells in vitro are freed from the complex control systems that exist in vivo, it is worthwhile to attempt to determine to what extent in vitro experiments mirror the same processes as they occur in vivo. In order to address this problem we decided to measure the rate of TGFA turnover in vivo in various fat depots under ad libitum feeding and sedentary conditions. The method chosen to accomplish these goals was to enrich adipose tissue TGFA with a fatty acid containing an odd number of carbon atoms, undecanoic acid (C-11), by adding it to the diet in the form of triglyceride as suggested by Campbell and Hashim (14). This fatty acid is not synthesized in the body nor recirculated back to adipose tissues to any extent in the form of VLDL once it has been mobilized (15). These properties permit C-11 to be used as a tool for the determination of rates of TGFA mobilization from adipose tissues in vivo.

MATERIALS AND METHODS

Animals used in this study were male Sprague-Dawley rats (Holtzman Co., Madison, Wisconsin) of two different initial body weights, 84 ± 1 and 333 ± 2 g (mean ± SEM), representing initial ages of approximately 4 and 20 weeks, respec-

tively. These two groups of rats are comparable with respect to both age and weight to those we have used previously in in vitro lipolytic studies (4,5). For the first four weeks of the study rats were fed a powdered semisynthetic diet composed of 6% corn oil (Mazola Corn Oil, Englewood Cliffs, New Jersey), 14% triundecanoin (P.V.O. International, Boonton, New Jersey) and 80% cholesterol-free diet (ICN, Cleveland, Ohio) after the method of Campbell and Hashim (14) to load the adipose tissues with C-11. Following the initial 4-wk feeding period, triundecanoin was withdrawn from the diet and replaced with corn oil to maintain the caloric composition of the diet constant.

Rats were killed by decapitation in groups of five at the time that triundecanoin was removed from the diet and at intervals of 1, 2, 3 and 4 weeks following its withdrawal. Fat cells were isolated from the epididymal (Epi), perirenal (PR), subcutaneous (SC) and mesenteric (M) depots by digestion with collagenase (1 mg/ml) in Krebs-Ringer bicarbonate buffer containing bovine serum albumin (10 mg/ml) and glucose (1 mg/ml). Tissues were incubated for 1 hr at 37 C, filtered through nylon chiffon with a diameter of approximately 25 μm and washed three times with fresh buffer (3-5). One-ml aliquots of the washed cell suspensions were extracted directly in 19 ml of isopropanol (3-5). This extraction procedure, which has been used widely for the extraction of plasma for determination of both cholesterol (16) and triglycerides (17), has proven in our hands to provide quantitative extractions of both of these lipids from adipocyte suspensions when compared to a more classical method such as extraction with 2:1 chloroform:methanol (18). An aliquot of each cell suspension also was used for determination of mean cell diameter utilizing a microscope equipped with a calibrated ocular micrometer (3-5). The triglyceride content per cell in both μg and pmoles was calculated from the mean cell diameter and the variance of the cell size distribution (3-5) assuming that the density of cellular triglyceride is similar to triolein (0.915 g/ml).

Fatty acid methyl esters of adipocyte TGFA were prepared from an aliquot of each extract using Meth-Prep II (Applied Science Laboratories, Inc., State College, Pennsylvania). The methyl esters were separated on a 6-ft column of 10% Silar 10C on Gas Chrom Q (Applied Science Laboratories, State College, Pennsylvania) in a Perkin Elmer model 3920 gas chromatograph equipped with flame ionization detectors. Integration of peak areas was performed on an HP 3390A integrator (Hewlett-Packard, Avondale, Pennsylvania). In order to assure that all peaks eluted in a reasonable time and with good resolution, columns were operated at a nitrogen flow rate of 30 ml/min with temperature programming of 80 C for 8 min followed by an increase of 4 C/min to a final temperature of 180 C. Column and instrument performance were checked daily with a series of quantitative standard mixtures containing all fatty acid methyl esters expected and ranging from C-8 to C-24 (Mixtures KB, KC, K-102 and K-108, Applied Science Laboratories, State College, Pennsylvania). Fatty acid composition was converted from weight percent to mole percent utilizing appropriate factors for the molecular weight of each fatty acid. In addition to this means of data expression it became obvious, for reasons to be discussed below, that in order to make valid comparisons between depots or between rats of different ages it was necessary to calculate the actual content (pmoles) of C-11 in the cells at each time point. This was done by multiplying the mole fraction of C-11 present (determined by gas chromatography) by the number of pmoles of total TGFA present per cell (calculated from the mean cell diameter and the variance of the cell size distribution) as described above with the additional assumption that the molecular weight of cellular triglyceride was similar to that of triolein.

Curve fitting was performed on an Apple II+ microcomputer using software developed by Interactive Microware (State College, Pennsylvania). Statistical analyses for the slope and intercepts of the fitted lines were performed on the raw data (values for individual animals at each time point) utilizing a program developed for computing these values and the 95% confidence intervals (19). Raw data were used rather than mean data at each time point so that the calculated regression lines would reflect the variability in all the data and thus help to prevent declaring statistical differences where none existed. Values were considered statistically different with $p < .05$ if the 95% confidence limits did not overlap.

RESULTS

Growth curves for the young and old rats studied are shown in Figure 1. It may be observed that both groups grew steadily throughout the entire period of study and that within each age group the growth rate was not affected by the feeding of triundecanoin. At the end of the 4-wk feeding period the mole percent of C-11 found in the adipose tissues within each age group averaged 40 ± 1 and 32 ± 1% for the young and old groups, respectively.

The results relating disappearance of C-11

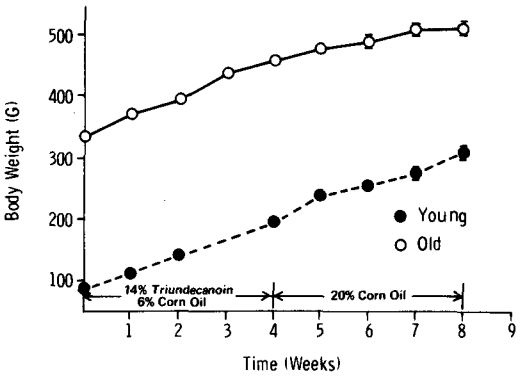

FIG. 1. Weight gain in young and older rats during the experimental feeding period with triundecanoin and following its removal from the diet and replacement with corn oil. Data are expressed as the mean ± the standard error of the mean.

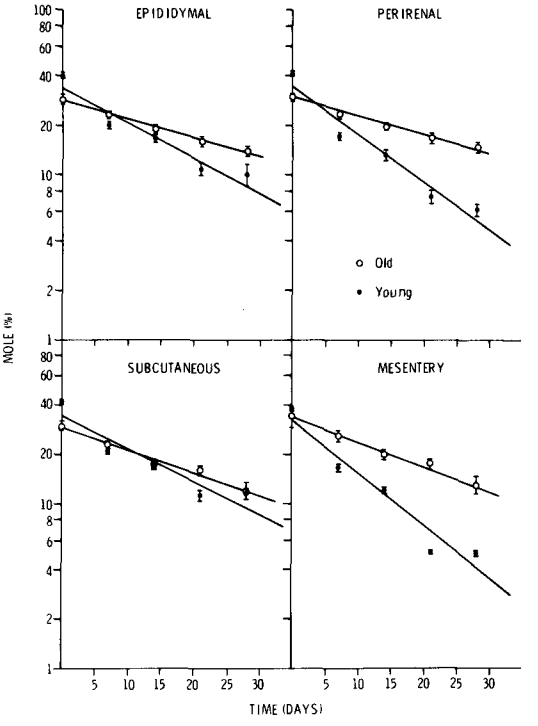

FIG. 2. Rate of disappearance of C-11 from adipocytes of young and older animals based on content expressed as the mole fraction contained in the cellular triglyceride in the four adipose tissue depots examined. Data are reported as the mean ± the standard error of the mean. Lines are the calculated least squares regression lines. Coefficients for the lines are provided in Table 1.

from adipose tissues in both the young and older groups are depicted in Figure 2. In the young group the fractional loss of C-11 varied from 4.5 to 7.3%/day, whereas in the older group has ranged from 2.6 to 3.4%/day (Table 1). From the results reported in Table 1, mobilization was significantly more rapid from PR and M adipocytes than from Epi and SC adipocytes in the younger animals. By contrast, no differences were apparent among the depots in older animals. In addition, mobilization was significantly slower in all depots of the older rats except for the SC depot. Data expressed in this manner, however, could be misleading because the mole fraction of C-11 in adipocytes decreases not only as a result of mobilization, but also as a result of continuing cell growth and accretion of cellular TGFA throughout the experimental period. Thus cellular C-11 is diluted at variable rates in both age groups due to differences in the rate of adipocyte hypertrophy in the different depots. This dilution effect results in an overestimation of the rate at which C-11 decreases as a result of TGFA mobilization.

To resolve this problem, the data were recalculated to reflect the actual pmoles of C-11 within the adipocytes at each time point. These

TABLE 1

Fractional Rate of Release of Undecanoic Acid from Adipose Tissues: Mole Percent Basis

Group	Depot	Slope (days^{-1})[a]	t½ (days)[b]	Intercept (mole %/cell)[b]
Young	Epi	-0.049 ± 0.009	14 (12-17)	33 (28-39)
	PR	-0.067 ± 0.009	10 (9-12)	34 (29-40)
	SC	-0.045 ± 0.008	15 (13-19)	34 (30-39)
	M	-0.073 ± 0.010	9 (8-11)	32 (27-38)
Old	Epi	-0.026 ± 0.006	27 (22-35)	28 (26-31)
	PR	-0.026 ± 0.006	27 (22-35)	30 (27-33)
	SC	-0.032 ± 0.008	22 (17-29)	30 (26-34)
	M	-0.034 ± 0.009	20 (16-28)	34 (30-40)

[a]Data expressed as the mean ± 95% confidence limits.

[b]Data are reported as the mean. Values in parentheses are the lower and upper 95% confidence limits, respectively.

data are reported in Figure 3. Data for these groups were compared statistically by comparing the 95% confidence limits for the slopes and intercepts of the fitted least squares lines in Figure 3 and the results reported in Table 2. Data for the rates of mobilization using this form of expression range from 2.6 to 4.9%/day in the young animals and from 1.6 to 2.3%/day in the older animals. These rates are lower than for the respective figures in Table 1. This results from the fact that the data in Table 2 now reflect the rate of disappearance of C-11 from the constituent adipocytes. Indeed, it is possible that the rate of mobilization of C-11 is now underestimated to some extent, because the statistical chance for a molecule of C-11 to be mobilized should decrease as the cellular TGFA pool increases. Using the data in Table 2, no differences were observed among depots with regard to the rates of mobilization in the younger group. Likewise, and in contrast to the data in Table 1, no significant differences were noted within each depot as a result of age, although the average rates for each depot were uniformly lower in the older animals. These results underscore the critical importance of the manner of data expression when drawing conclusions regarding the turnover of TGFA in adipose tissues of growing rats.

It is also of interest to examine the y-intercepts which are listed in Tables 1 and 2. In the data of Table 1, the amount of C-11 present in the cells at the end of the initial enrichment phase averaged 32% of the total TGFA with no differences observable as a result of either anatomical location or age. When the data are expressed on the basis of pmoles C-11 per cell (Table 2), the y-intercepts in the younger animals were not different among the depots except for the M depot which contained significantly less C-11 than the other three depots. This difference results from the fact that, while

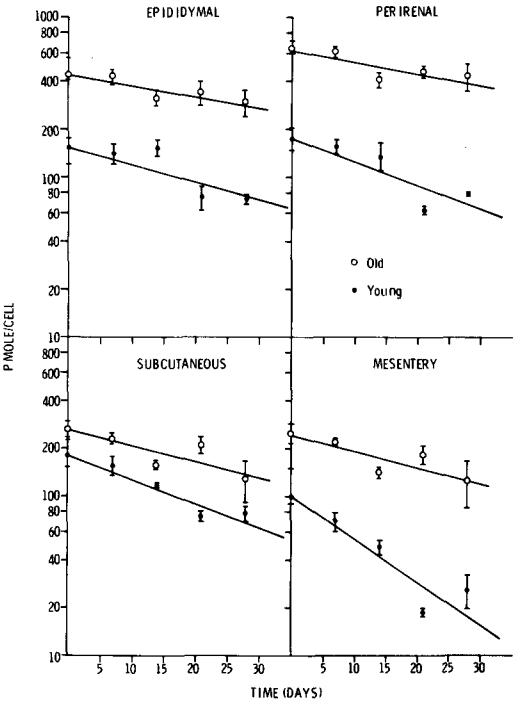

FIG. 3. Rate of disappearance of C-11 from adipocytes of young and older animals based on content expressed as pmoles of C-11 per cell in the four adipose tissue depots examined. Data are expressed as the mean ± the standard error of the mean. Lines are the calculated least squares regression lines. Coefficients for the lines are provided in Table 2.

the content of C-11 based on mole percent was not different, the cells in the M depot contained 20-45% less TGFA than those in the other depots. This observation is consistent with other cell size data from our lab (4,5). Data for the initial content of C-11 (pmoles/cell) in the older animals were all significantly greater than for

TABLE 2

Fractional Rate of Release of Undecanoic Acid from Adipose Tissues: PMole per Cell Basis

Group	Depot	Slope (days^{-1})[a]	t½ (days)[b]	Intercept (pmole/cell)[b]
Young	Epi	-0.026 ± 0.016	27 (16-69)	155 (118-202)
	PR	-0.034 ± 0.014	20 (14-35)	178 (140-226)
	SC	-0.034 ± 0.012	20 (15-32)	180 (147-221)
	M	-0.049 ± 0.022	14 (10-16)	76 (52-110)
Old	Epi	-0.018 ± 0.014	38 (22-173)	447 (351-573)
	PR	-0.016 ± 0.009	43 (28- 99)	626 (534-735)
	SC	-0.023 ± 0.012	30 (20- 63)	265 (217-324)
	M	-0.023 ± 0.012	30 (20- 63)	243 (198-299)

[a]Data are expressed as the mean ± 95% confidence limits.

[b]Data are reported as the mean. Values in parentheses are the lower and upper 95% confidence limits, respectively.

the respective depots in the younger animals, in spite of the fact that the mole percent of the total TGFA represented by C-11 was less. In addition, the content of C-11 expressed as pmoles/cell in both SC and M adipocytes was significantly less than that in the Epi and PR depots because of the smaller size of the cells in the former two depots.

It is difficult to draw any conclusion about the absolute rate of mobilization of TGFA from these data because the adipocytes are not in a steady state as far as pool size is concerned, i.e. adipocyte sizes continued to increase in size during the entire experimental period (data not shown). Such a phenomenon has been reported previously by this investigator (3-5). It may be noted, however, that the amount of undecanoic acid present at zero time in Figure 3 is proportional to cell size. Therefore, since none of the fractional mobilization rates were significantly different, it probably is safe to conclude that the larger cells have an increased absolute rate of mobilization on a molar basis.

DISCUSSION

The results of this study confirm previous reports of the utility of using triundecanoin for the study of TGFA turnover in adipose tissue (15,20-21). From comparison of the growth curves for the two groups of animals it is apparent that the addition of triundecanoin to the diet produced no adverse effects on the growth rate compared to the control period following its removal. These data are consistent with previous results in both rats and dogs which showed no effect of C-11 on growth (15,21). It also has been reported in dogs that feeding C-11 resulted in the accumulation of longer odd-chain fatty acids that apparently resulted from chain elongation of the C-11 (21). This observation, however, either has not been made or not been reported in rats (15,20). No evidence of chain elongation was apparent from the fatty acid profiles of any of the adipose tissue extracts in the present study. The reason(s) for this species difference is (are) not readily apparent.

The major advantage of using C-11 for these studies is the fact that only minimal recycling in the form of circulating triglycerides occurs back to the adipose tissues (21). This allows one to determine directly the rate of TGFA mobilization from these depots. This is not the case when longer chain fatty acids are traced because these molecules may be recycled significantly and result in slower apparent rates of mobilization (1). Another advantage of this experimental design is the fact that the total amount of fat in the diet was not changed throughout the course of the study. This principle of maintaining dietary lipid constant throughout the course of turnover studies has been overlooked in some studies in which safflower oil has been used in the diet to enrich the adipose tissue TGFA with linoleic acid (22, 23). At the end of the enrichment period, animals were switched to a fat-free diet and the rate of linoleic acid depletion from this tissue determined. The effect of removal of lipid from the diet on TGFA turnover was not studied (22,23).

The preliminary feeding period for C-11 of 4 wk seemed adequate to label the adipose tissue depots in animals of both ages. In our study the average C-11 content in cellular TGFA on a molar basis was 32%. This may be compared to a value of about 23% for a similar group of control rats by Askew et al. (20) and 30% reported by Campbell and Hashim (15). The latter investigators fed their animals a diet enriched with about 1/3 more C-11 than that used by either Askew et al. (20) or ourselves.

One interesting aspect of the fatty acid composition at the end of the enrichment period is that the fatty acid content of C-11 on a mole percent basis was the same in all depots regardless of age. As in previous studies from this lab, cell sizes among the depot of rats of the same age were not homogeneous (4,5,24). Likewise, adipocytes from the same depots were larger in the older animals than in the younger animals. Thus, even though the composition in all these depots was similar, the actual mass of C-11 differed and was proportional to the total amount of triglyceride stored within the cells. These results are compatible with previous results from this lab regarding both lipoprotein lipase (LPL) activity in these depots and the ability of these tissues to remove circulating TGFA in the form of chylomicrons (24). In this latter report as well as in the present study, the sizes of Epi and PR cells were larger than those of the SC and M depots. The distribution of LPL activity within the tissues of these depots also was different. For any given adipocyte size, Epi and PR tissues had more of the total tissue LPL located outside the adipocytes and potentially at the capillary endothelium than that observed for the SC and M depots (24). As a result, it is not surprising that in the present study the larger cells, i.e., Epi and PR, contained a greater mass of C-11 than that found in the other two depots. These differences in the mass of C-11 per cell among the depots were accentuated in the older animals.

Data presented in Table 1 indicate a half-time of disappearance of C-11 from the young rats ranging from 9-15 days and in the older rats

from 20-27 days. Our data on the PR depot of 10 days is similar to a value of 12 days in rats of a similar age reported by Campbell and Hashim (15) while our value of 27 days in the older rats is almost identical to that of rats of a similar age reported by Askew et al. (20). In spite of the fact that our data agree with previous data in the literature and the fact that within the data contained in Table 1 a number of potentially interesting statistical differences exist, in our judgment these values overestimate the "true" rates of mobilization because the cells were continuously enlarging at different rates in the various depots. Because the molar composition of cellular C-11 is decreased by the acquisition of an increased mass of TGFA as well as by mobilization and because the cells of different depots enlarge at different rates (4,5, 22), one can compare these data only on the basis of pmoles of C-11 mobilized. When this is done as in Table 2, all of the potential differences between the depots at both ages disappear, as does the effect of age within each of the depots. Indeed, as indicated earlier, these data may even underestimate the rate of mobilization due to a decreased probability of a given molecule being mobilized when the pool size is increased. It is thus apparent that the way data are expressed in these types of studies can dramatically affect the interpretation(s). To this investigator's knowledge, this is the first report in which the vitally important aspect of cellular enlargement has been taken into account when interpreting these types of data.

One of the objectives of this study was to correlate the in vitro rates of release reported in a previous publication (4,5) with those obtained in in vivo experiments. In the in vitro studies with respect to basal and norepinephrine-stimulated lipolysis, mobilization was expressed on the basis of glycerol release. In that study an age-related decrease in norepinephrine-stimulated glycerol release was observed in all depots with the exception of Epi adipocytes. In addition, it was observed that the degree of re-esterification of hydrolyzed TGFA was increased in Epi and PR adipocytes with age. In our present in vivo studies the differences in fatty acid mobilization with respect to age were minimal in all depots. The fact that no statistically significant age-related differences were observed in vivo may indicate that the turnover of TGFA under conditions of a normal ad libitum diet is not under the control of the sympathetic nervous sytem and is more related to basal release in vitro which does not differ between depots (4,5). It also may be true that even under the extreme condition of starvation lipolysis may not be directly related to sympathetic stimulation. When rats were starved for 72 hr, the decrease in cell sizes in the different depots of older animals was related to initial cell size rather than to the lipolytic sensitivity to norepinephrine (4,5). Other factors such as possible differences in the antilipolytic sensitivity between cells of different depots also may play a prominent role in the in vivo situation.

The present data indicate that, although there are no differences in the fractional rate of mobilization of C-11 from adipocytes that may be related to either anatomical location or age, the fact that the pool sizes in the different depots vary results in the release of a greater mass of fatty acids per unit time from those depots containing the largest cells. Because these studies were performed in ad libitum-fed rats it appears as though the rate of release of fatty acids under both fed and fasted conditions may be related to the mass of stored TGFA.

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Dietary Fats Containing Concentrates of *cis* or *trans* Octadecenoates and the Patterns of Polyunsaturated Fatty Acids of Liver Phosphatidylcholine and Phosphatidylethanolamine

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ABSTRACT

The effects of the mixed *cis*-18:1 isomers and mixed *trans*-18:1 isomers present in partially hydrogenated soybean oil (PHSO) upon the patterns of polyunsaturated fatty acids (PUFA) in liver phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were studied in rats fed concentrates of *cis*-18:1 or *trans*-18:1 isomers isolated as triacylglycerides from PHSO. The *cis*-18:1 and *trans*-18:1 concentrates were fed at levels equal to those present in PHSO fed at 17.9% of the diet. All diets contained the required amounts of both linoleic and linolenic acids. The *trans*-18:1 concentrate was found to suppress the levels of 20:4ω6 and 20:3ω9, and to increase the levels of 18:2ω6 and 20:5ω3 in PC and PE. The *cis*-18:1 concentrate suppressed 20:4ω6 in PC, 20:5ω3 in PC and PE, and 18:2ω6 in PC, but increased the levels of 20:4ω6 in PE, and 20:3ω9 in PC and PE. The *cis*-18:1 concentrate was more effective than the *trans* concentrate in suppressing 22:6ω3. The *trans*-18:1 concentrate was more effective in suppressing 20:4ω6. The *trans*-18:1 isomers appear to modify PUFA metabolism by inhibition of PUFA synthesis, whereas the *cis*-18:1 isomers appear to compete with 2-position fatty acyl transfer and to inhibit ω3 PUFA acylation.
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INTRODUCTION

Partial hydrogenation of plant oils produces a broad range of positional isomers (Δ7 to Δ16) of both *cis* and *trans* octadecenoic acids (*c*-18:1 and *t*-18:1) (1,2) and minor amounts of geometric isomers of linoleic acid (2,3). Feeding partially hydrogenated plant oils to rats has been shown to suppress the arachidonic acid content of lipids in several tissues (4-6). Recently we have shown that arachidonic acid of liver phosphatidylcholine (PC) is particularly suppressed in rats fed partially hydrogenated soybean oil (PHSO) while liver phosphatidylethanolamine (PE) arachidonate is considerably less affected (5). The suppression of arachidonic acid presumably is caused by one or more of the octadecenoic acid isomers, because the predominant geometric isomers of linoleic acid have been shown to have no effect on the arachidonic acid content of rat liver (7). Indeed, several of these octadecenoates have been shown to inhibit Δ5 and Δ6 desaturases in rat liver microsomes (8,9).

Ideally, the effects of dietary intake of each of the individual octadecenoates on polyunsaturated fatty acid (PUFA) metabolism should be studied. However, preparative separation of the positional isomers currently is unfeasible, and synthesis of adequate amounts of the many isomers would require several years. As a first step, we decided to study the effects of the *cis* and *trans* isomers as groups because concentrates of these could be prepared by fractional crystallization of PHSO. Concentrates of ethyl esters with higher purity than used in this experiment could have been obtained by fractional crystallization of ethyl esters of PHSO; however, preliminary measurements showed that only about 60% of the ethyl esters of such *trans*-octadecenoate concentrates were absorbed.

MATERIALS AND METHODS

The *trans* concentrate was prepared by dissolving 5 kg PHSO in 90 liters of warm acetone and crystallizing the triacylglycerols relatively rich in *t*-18:1 isomers at room temperature. The yield was 16%, and fatty acid analysis revealed a *t*-18:1/*c*-18:1 ratio of 7.0, compared with a ratio of 1.8 in the original PHSO. The *cis* concentrate was the filtrate of a −20 C precipitation of PHSO in acetone (1:18, v/v). The yield was 9%, and these triacylglycerols had a *c*-18:1/*t*-18:1 ratio of 1.95 compared to 0.55 in PHSO.

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The *trans* concentrate contained 41% *t*-18:1 and 5.8% *c*-18:1. The *cis* concentrate contained 56% *c*-18:1 and 28% *t*-18:1.

Four-week-old male Sprague-Dawley rats in groups of five were fed five semipurified diets based upon a diet designed for study of essential fatty acid (EFA) nutrition (10). In addition to 23 wt % fat, the diets contained (wt %) sucrose, 42.2; vitamin-free casein, 25; nonnutritive fiber, 4; Williams-Briggs salts, 3.5; vitamin mix, 1 (10); choline chloride mix, 1 (10); and DL-methionine, 0.3. All diets were isocaloric and contained mixed fats to provide 3.45% of calories as linoleate (estimated requirement = 1.0% of cal [11]) and 1.1% of cal of linolenate (estimated requirement = 0.5% of cal [12,13]). The CONTROL diet contained 21.2% beef tallow, 1.0% corn oil and 0.84% linseed oil, which provided minimum proportions of *c*- and *t*-18:1 isomers other than oleic acid. The PHSO diet contained 17.9% PHSO, 2.53% beef tallow, 1.61% corn oil and 0.99% linseed oil, which provided moderate levels of *c*- and *t*-18:1 isomers. The TRANS diet contained 20.02% *trans* concentrate, 1.99% corn oil and 0.99% linseed oil, which provided approximately the same amount of *t*-18:1 as did the PHSO diet. The CIS diet contained 10.3% *cis* concentrate, 10.9% beef tallow, 0.90% corn oil and 0.93% linseed oil, which provided the same amount of non-endogenously synthesizable *c*-18:1 as did the PHSO diet (*c*-9-18:1 and *c*-11-18:1 are endogenously synthesized). The 1.5-CIS diet contained 15.42% *cis* concentrate, 5.75% beef tallow, 0.86% corn oil and 0.97% linseed oil. The 1.5-CIS diet was fed to emphasize the effects of non-endogenously synthesized *c*-18:1 isomers. The fatty acid composition of the total fat in each diet given in Table 1 was calculated from the analyses of the individual fat components and the analysis of the exhaustively extracted lipid from the non-fat portion of the diet. Diets were refrigerated and fresh portions fed daily to minimize autoxidation. Each rat was weighed before and after the 18-day experimental period.

The rats were anesthetized with diethyl ether and killed by heart puncture. Tissues were removed rapidly and stored at −20 C until analyzed. Tissues were extracted with chloroform/methanol (2:1, v/v) (14), and PC and PE were isolated by thin-layer chromatography with chloroform/methanol/glacial acetic acid/water (66:33:6:3, v/v/v/v), and transesterified with 14% BF_3/methanol at 75 C for 30 min. Absolute concentrations of PC and PE were not determined because even vast changes in dietary fat have no effect on membrane phospholipid class distribution (4,15). Fatty acid methyl esters were analyzed using a 50 m × 0.2 mm i.d. wall-coated free fatty acid phase (FFAP) fused silica capillary column (Scientific Glass Engineering, Inc., Austin, Texas) with flow of 0.45 ml/min and split ratio of 1:64. Total *c*-18:1 and total *t*-18:1 were separated on Silica Gel H plates impregnated with 2% $AgNO_3$, an internal standard was added to each fraction, and each mixture was analyzed by gas chromatography (GC). Individual positional isomers of *c*- and *t*-18:1 were measured by

TABLE 1

Fatty Acid Compositions of Total Mixed Dietary Fats in the Five Diets[a]

	Diet[b]				
Fatty acid	CONTROL	PHSO	TRANS	CIS	1.5-CIS
14:0	2.6	0.3	0.1	1.4	0.8
16:0	26.6	12.9	14.3	16.0	10.6
16:1	3.3	0.6	0.1	1.8	1.1
18:0	15.7	13.3	31.4	9.4	6.0
t-18:1	2.2	36.0	36.2	14.6	20.3
c-18:1	40.4	26.5	8.3	47.8	50.4
c-18:1[c]	1.04	10.3	3.6	11.4	17.1
18:2ω6	6.7	6.6	6.7	6.6	6.6
18:2 isomers	0.3	1.8	0.5	1.5	2.0
18:3ω3	2.1	2.1	2.1	2.1	2.1
20:0	0.2	0.25	0.7	0.1	0.1

[a]Values represent percent of dietary fatty acids.

[b]Diets contain the following fats as the principal dietary fat component: CONTROL, beef tallow; PHSO, partially hydrogenated soybean oil; TRANS, *t*-18:1 concentrate isolated from PHSO; CIS, *c*-18:1 concentrate isolated from PHSO; 1.5-CIS, 50% more *c*-18:1 concentrate than used in CIS diet.

[c]*c*-18:1 Isomers minus *c*9- and *c*11-18:1 which are synthesized endogenously.

ozonization of the *cis* and *trans* fractions, thermal cleavage of the ozonides and separation of the aldehyde esters on the FFAP capillary column. Values for aldehyde esters were corrected for number of ionizable carbon atoms (16). Isomers from Δ7 through Δ16-18:1 were individually measurable. The results were checked by FFAP capillary GC using FFAP as liquid phase, which separates individually many of the *c*-18:1 isomers (Δ11-Δ16) and a few of the *t*-18:1 isomers (Δ14-Δ16). Values obtained by these methods agreed within 10%. Significance of differences between means was measured by Students' t-test (17).

RESULTS

Final weights of rats (range 188-226 g) and weight gains (range 101-130 g) of the four experimental groups over the 18-day study were not significantly different from those of the CONTROL group.

PUFA in PC of Liver

The effects of dietary PHSO and of the *c*-18:1 or *t*-18:1 concentrates from PHSO upon the fatty acid composition of liver PC are shown in Table 2. The PHSO and TRANS diets suppressed 20:4ω6 to half the value of the CONTROL group and increased 18:2ω6 and 20:5ω3, but effects upon other fatty acids were minor. The TRANS diet had opposite effects upon 20:4ω6 and 20:5ω3, the major products of Δ5 desaturation, indicating a more specific mechanism than suppression of Δ5 desaturation. Effects of the TRANS diet were more pronounced than those of the PHSO diet, even though they caused equal accumulation of *t*-18:1 in PC. The CIS diet moderately suppressed 20:4ω6, perhaps due to the presence of some *t*-18:1 (see Table 1), but strongly suppressed 22:6ω3, and, in sharp contrast to the PHSO and the TRANS diets, caused a modest suppression of 18:2ω6. The product/precursor ratio, 20:4ω6/18:2ω6, was reduced by PHSO and TRANS diets to 44% and 33% of the control value, respectively, but was unaffected by the CIS diet. The 20:3ω9 was increased 3-fold by the CIS diet and was suppressed by the TRANS diet. Effects of the CIS diet were accentuated by the 1.5-CIS diet, except for the suppression of linoleate.

Individual isomers of *c*- and *t*-18:1 measured in the dietary fats and in the liver PC from each group are given in Table 3. In all groups, discrimination against incorporation of the *c*10-18:1 and *t*10-18:1 isomers into PC occurred, when compared to their dietary levels, a phenomenon reported by others (18,19). The *t*12-18:1 and *t*14-18:1 isomers accumulated in liver PC 2- to 3-fold above the dietary source in all groups. The *c*12-18:1 isomer, however, accumulated in PHSO fed rats, but was discriminated against in rats fed the individual concentrates. Apparently, the combination of *c*-18:1 and *t*-18:1 isomers can have a synergistic effect on *c*12-18:1 accumulation. These data do not indicate which of the isomers are related to changes of PUFA content. Correlation analysis between the level of each isomer and the

TABLE 2

Effects of *Cis* and *Trans* Octadecenoate Concentrates on the PUFA Patterns of Liver PC[a]

Fatty acid	Diet[b]				
	CONTROL	PHSO	TRANS	CIS	1.5-CIS
16:0	17.7 ± 2.2	13.1 ± 1.3[c]	15.0 ± 1.2[d]	21.3 ± 0.8[c]	22.1 ± 1.6[c]
16:1	1.2 ± 0.3	2.8 ± 0.5[c]	2.9 ± 0.1[c]	1.7 ± 0.3[d]	1.9 ± 0.4[c]
18:0	22.4 ± 2.0	16.6 ± 1.5[c]	18.7 ± 1.6[c]	17.9 ± 1.6[c]	16.6 ± 1.8[c]
c-18:1[e]	9.8 ± 0.6	10.9 ± 0.5[c]	9.1 ± 0.9	17.0 ± 0.15[c]	18.5 ± 2.5[c]
t-18:1[e]	1.5 ± 0.2	11.8 ± 1.2[c]	11.4 ± 0.4[c]	3.0 ± 0.5[c]	3.3 ± 0.6[c]
18:2ω6	10.1 ± 1.1	13.3 ± 0.6[c]	16.0 ± 0.8[c]	8.8 ± 1.1[d]	8.9 ± 0.5[d]
20:3ω9	0.67 ± 1.8	0.45 ± 0.07[d]	0.39 ± 0.10[c]	1.9 ± 0.2[c]	2.6 ± 0.4[c]
20:3ω6	1.8 ± 0.2	2.1 ± 0.4	2.2 ± 0.2[c]	2.3 ± 0.2[c]	2.2 ± 0.3[d]
20:4ω6	20.5 ± 1.7	12.0 ± 0.8[c]	10.9 ± 1.2[c]	17.1 ± 1.2[c]	14.4 ± 2.1[c]
20:5ω3	1.0 ± 0.3	1.6 ± 0.3[c]	2.0 ± 0.3[c]	0.8 ± 0.2	0.7 ± 0.2
22:5ω3	0.81 ± 0.31	0.96 ± 0.27	1.01 ± 0.21	0.75 ± 0.20	0.40 ± 0.07[c]
22:6ω3	7.2 ± 0.6	6.8 ± 1.1	6.1 ± 0.8[d]	4.1 ± 0.6[c]	3.7 ± 0.9[c]

[a]Values represent mean ± S.D. of percentages for 5 animals. The differences between the sum of values given and 100% represent several minor fatty acids not shown.

[b]For diet abbreviations, see Table 1.

[c,d]Significantly different from control value, $p < 0.01$, $p < 0.05$, respectively.

[e]Determined after separation of methyl esters by argentation chromatography. Values represent mean ± S.D. for 3 or 4 animals.

depression of 20:4ω6 in the liver phospholipids (PL) of PHSO-fed rats (5) revealed significance only for *c*12-18:1 and *c*13-18:1, and synthetic *c*12-18:1 fed alone depressed 20:4ω6 of liver PL. In the present study, effects of other isomers overrode those of *c*12-18:1 and *c*13-18:1.

The content of total *t*-18:1 isomers in the PHSO diet and in the TRANS diet were equal, but the TRANS diet contained more *t*-18:1 isomers in which the double bonds were near the methyl end of the molecules, because of segregation in the recrystallization procedure. Unequal distribution of *t*-18:1 isomers in the PHSO and TRANS diets may have contributed to the greater effects of the TRANS diet upon PUFA levels.

PUFA in PE of Liver

Effects of dietary fats on PUFA composition of PE were generally similar to those for PC, but some distinct differences were found (Table 4). The PHSO and TRANS diets suppressed 20:4ω6 of liver PE to a lesser degree than in PC. The CIS diet increased the 20:4ω6 content of PE to 125% of the control value, whereas it decreased PC 20:4ω6 significantly. This accumulation was not further increased by the 1.5-CIS diet.

DISCUSSION

Mixed *c*-18:1 isomers and mixed *t*-18:1 isomers isolated from PHSO had different yet significant effects upon PUFA patterns in liver PC and PE. Position and geometry of unsaturation in fatty acids are known to affect metabolic reactions (8,9,20,21). The effects of mixed *c*-18:1 isomers and mixed *t*-18:1 isomers were easily distinguishable even though concentrates, not pure substances, were fed.

The TRANS, CIS and 1.5-CIS diets contained about equal proportions of total 18:1 isomers, excluding endogenous *c*9- and *c*11-18:1 isomers. The TRANS and PHSO diets suppressed 20:4ω6 about equally, but the CIS diet suppressed 20:4ω6 to a lesser degree in PC and not at all in PE. Therefore, suppression of 20:4ω6 in liver PC and PE by PHSO must be related to the *trans* isomers present in PHSO.

Although mixed *t*-18:1 and mixed *c*-18:1 isomers suppressed 20:4ω6 in liver PC, the mechanisms of suppression by these mixtures appear to be different. The *t*-18:1 isomers probably inhibit PUFA biosynthesis because the TRANS diet suppressed the product/precursor ratios 20:4ω6/18:2ω6 and 22:6ω3/20:5ω3 by 65% and 55% in both PC and PE (see Tables 2 and 4). The CIS diet, however, did

TABLE 3

Isomeric Composition of Dietary and Liver PC Octadecenoates[a]

	Diet[b]									
	CONTROL		PHSO		TRANS		CIS		1.5-CIS	
Isomer	Diet	PC	Diet	PC	Diet	PC	Diet	PC	Diet	PC
c-7	0.5	nd	2.3	1.7	2.1	1.6	1.7	0.9	2.2	0.7
c-8	0.5	6.0	5.1	4.3	3.7	3.7	5.4	3.2	7.2	2.7
c-9	93.0	75.3	53.7	44.0	49.6	65.0	69.1	72.9	57.1	78.9
c-10	0.2	nd	8.3	3.2	5.2	1.6	7.6	1.8	10.7	0.5
c-11	4.5	17.3	10.0	15.6	8.6	9.7	6.5	11.2	8.7	7.6
c-12	0.5	1.3	11.5	19.6	10.6	8.4	7.0	6.5	10.5	5.9
c-13	0.7	3.0	4.4	5.4	6.2	3.8	1.8	1.8	2.2	2.7
c-14	nd	0.4	2.2	3.2	5.2	2.1	0.6	0.6	0.8	0.7
c-15	nd	nd	1.3	2.3	4.5	2.2	0.3	0.7	0.4	0.5
c-16	nd	nd	1.1	1.5	4.2	1.8	0.1	0.4	0.2	0.3
t-7	nd	nd	2.6	1.8	1.7	2.3	2.2	3.3	2.4	2.6
t-8	nd	4.0	9.1	6.5	5.9	5.4	7.2	5.7	7.8	6.4
t-9	10.4	22.0	17.1	13.3	12.4	10.9	17.3	16.0	18.5	18.5
t-10	14.9	5.0	23.4	5.2	21.7	7.0	22.9	5.7	24.8	6.3
t-11	64.5	50.0	18.1	14.7	19.8	13.4	24.0	18.7	23.1	18.8
t-12	5.0	16.0	12.8	32.5	15.6	27.0	15.1	23.3	11.6	23.3
t-13	5.0	3.0	8.9	10.2	11.0	10.7	6.5	10.0	6.9	8.2
t-14	nd	nd	4.6	10.5	6.1	14.8	3.1	11.3	3.2	10.2
t-15	nd	nd	2.2	3.0	3.2	4.0	1.0	2.3	1.1	3.3
t-16	nd	nd	1.3	2.8	2.7	4.0	0.6	2.7	0.5	2.4

[a]Values represent percent of total *c*-18:1 or total *t*-18:1.

[b]For diet abbreviations, see Table 1.

nd, not detectable.

not affect PUFA biosynthesis because the 20:4ω6/18:2ω6 and 22:6ω3/20:5ω3 ratios in both PC and PE were nearly equal to those in CONTROL animals.

The *c*-18:1 isomers appear to affect PUFA composition in two ways. In PC, the *c*-18:1 isomers probably inhibit acyl transfer because total ω6+ω3 PUFA were significantly less in the liver PC of rats fed the CIS diet (34.2 ± 0.9, S.D.) or the 1.5-CIS diet (30.8 ± 2.5) than in rats on the CONTROL diet (42.6 ± 1.4), although all groups received equal levels of their dietary precursors. Comparable values for rats fed the TRANS diet were 39.0 ± 1.2, and for the PHSO were 37.3 ± 1.5. Clearly, the ω6+ω3 PUFA of liver PC decreased with increasing dietary *c*-18:1 isomers. The correlation coefficient between % of dietary isomeric *c*-18:1 (minus the Δ9 and Δ11 isomers synthesizable endogenously) and the total % of ω6+ω3 acids of liver PC was −0.96 with a slope of −0.663 and an intercept of 42.5% ω6+ω3 acids in liver PC.

The *c*-18:1 isomers are effective competitors for acylation at the 2-position of liver PC, the same position where PUFA are found almost exclusively. The *c*-18:1 isomers have been shown to have a 3-fold preference for the 2-positions over the 1-position of PC (5,18). In PE, where *c*-18:1 isomers do not occur preferentially in the 2-position (5,18), little difference was seen in the total ω6+ω3 PUFA between CONTROL (44.2 ± 1.7), and CIS fed animals (45.0 ± 14) or 1.5-CIS fed animals (45.3 ± 0.3). The *t*-18:1 isomers do not compete effectively with acylation of the 2-position because there is a 30-fold preference for acylation of *t*-18:1 isomers at the 1-position of liver PC and PE (18). Thus, the suppression of 20:4ω6 by *t*-18:1 isomers is probably not through competition in acylation.

The *c*-18:1 isomers may also affect PUFA composition by inhibition of acylation of ω3 fatty acids in a specific manner. Because ω6 and ω3 fatty acids compete for the 2-position, a decrease in available ω3 fatty acids should increase acylation of the ω6 fatty acids without affecting the total ω6+ω3 PUFA. This, indeed, was observed in PE of rats fed the CIS diet in which total ω3 fatty acids were decreased by 27% and total ω6 fatty acids were increased by 24% compared to CONTROL animals. The ω6/ω3 ratio increased from 1.64 ± 0.19 to 2.8 ± 0.3, but the total ω6+ω3 PUFA remained essentially unchanged (see Table 4). A similar decrease in ω3 fatty acids and increase in ω6/ω3 ratio (from 3.6 ± 0.2 to 4.9 ± 0.7) also was seen in PC of rats fed the CIS diet even though total ω6+ω3 PUFA was lower than that of the CONTROL animals. Thus, in PC, both competition by *c*-18:1 isomers for the 2-position and preferential inhibition of ω3 acylation appear to operate. In PE, only the latter appears to be a factor.

Suppression of 22:6ω3 in liver PC of rats fed the *c*-18:1 isomers indicates suppression of metabolism of linolenic acid and/or acylation by 22:6ω3. Thus, the utilization of 18:3ω3,

TABLE 4

Effects of *Cis* and *Trans* Octadecenoate Concentrates on the Fatty Acid Composition of Liver PE[a]

Fatty acid	Diet[b]				
	CONTROL	PHSO	TRANS	CIS	1.5-CIS
16:0	14.7 ± 1.0	11.0 ± 1.5[c]	10.8 ± 0.8[c]	12.4 ± 0.5[c]	12.9 ± 1.5[d]
16:1	0.6 ± 0.3	1.5 ± 0.3[c]	1.1 ± 0.6	0.55 ± 0.14	0.54 ± 0.07
18:0	26.5 ± 3.7	19.8 ± 1.3[c]	21.7 ± 1.8[d]	27.9 ± 0.9	26.2 ± 1.2
c-18:1[e]	5.8 ± 1.0	4.8 ± 0.9	3.9 ± 0.1[d]	7.5 ± 1.1	7.3 ± 0.5[d]
t-18:1[e]	1.3 ± 0.2	12.6 ± 1.7[c]	13.0 ± 0.1[c]	2.8 ± 0.1[c]	4.1 ± 0.9[c]
18:2ω6	2.7 ± 0.4	4.8 ± 0.65[c]	6.1 ± 0.6[c]	3.2 ± 0.6	3.3 ± 0.6[d]
20:3ω9	0.50 ± 0.25	0.22 ± 0.06[d]	0.39 ± 0.11	0.79 ± 0.11[d]	1.37 ± 0.36[c]
20:3ω6	0.75 ± 0.25	0.73 ± 0.1	0.92 ± 0.23	0.67 ± 0.10	0.75 ± 0.12
20:4ω6	23.1 ± 1.7	18.7 ± 0.6[c]	18.0 ± 0.4[c]	29.2 ± 1.2[c]	29.7 ± 1.2[c]
20:5ω3	1.2 ± 0.4	2.3 ± 0.6[c]	3.0 ± 0.4[c]	0.84 ± 0.22[d]	0.94 ± 0.29
22:5ω3	1.6 ± 0.8	2.0 ± 0.4	2.4 ± 0.5[d]	1.2 ± 0.2	0.85 ± 0.27[d]
22:6ω3	13.6 ± 1.8	16.2 ± 1.1[d]	15.5 ± 2.0	9.9 ± 1.0[c]	9.7 ± 1.6[c]

[a]Values represent mean ± S.D. of percentages for 5 animals. The differences between the sums of values given and 100% represent several minor fatty acids not shown.

[b]For diet abbreviations, see Table 1.

[c,d]Significantly different from control value, $p < 0.01$, $p < 0.05$, respectively.

[e]Values were determined after argentation chromatography of methyl esters and represent mean ± S.D. for 3 animals.

usually present in hydrogenated products in very low amounts, is impeded additionally by the *c*-18:1 generated in the hydrogenation process.

The suppression of 20:4ω6 in PC by dietary *t*-18:1 and *c*-18:1 isomers in PHSO may have additional physiological effects if syntheses of prostaglandins and other eicosanoids are affected (22). Diets containing other inhibitors of 20:4ω6 synthesis, such as linolenate (23) and *t,t*-linoleate (24), suppressed serum prostaglandin levels. Additional in vivo studies are needed with the individual positional *c*- and *t*-18:1 isomers to determine which isomers are the most effective modifiers of PUFA metabolism and perhaps of prostaglandin metabolism.

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Moderate Changes in Linoleate Intake do not Influence the Systemic Production of E Prostaglandins

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ABSTRACT

A pilot study was undertaken to determine if moderate changes in linoleate (18:2ω6) intake would modulate the prostaglandin E turnover concurrently with, or independently of, changes in the plasma prostaglandin (PG) precursor levels. Four adult male volunteers in good health were fed two controlled diets containing 35% of energy from fat, with either 10 (diet L) or 30 g (diet H) linoleate/day, 30 to 50 g saturated fatty acids/day, and the balance mainly monounsaturated fatty acids. All four subjects were consuming sufficient amounts of polyunsaturates before the study. Protein (13-14%) and carbohydrate (51-53%) contribution to total caloric intake was kept constant. The menu cycle was 7 days, and all diets were calculated to provide adequate amounts of nutrients known to be required by man when data were available. Plasma fatty acids were determined by gas-liquid chromatography, and the turnover of E prostaglandins was assessed by measuring the urinary output of the major metabolite of PGE_1 + PGE_2 (PGE-M). Whereas we found a clear correlation between 18:2ω6 intake and 18:2ω6 concentrations in the neutral lipid (P=0.007) and phosphoglyceride (P=0.012) fractions of plasma, arachidonate (20:4ω6) concentrations in those same plasma fractions did not respond significantly to changes in linoleate intake. Moreover, we could not detect an influence of moderate changes in dietary levels of 18:2ω6 on the systemic production of PGE as measured by the daily urinary output of PGE-M. *Lipids* 20:268-272, 1985.

INTRODUCTION

The mechanisms by which dietary lipid consumption affect mammalian and human physiology, specifically in the cardiovascular area, are very complex and not entirely understood (1-3). It has long been hypothesized that some of the beneficial effects associated with a relatively high consumption of polyunsaturated fatty acids (PUFA) could be due to diet-induced changes in the activity of the prostaglandin (PG) system (4,5). Linoleic acid (18:2ω6), an important lipid constituent of the Western diet, is the dietary precursor of mono- and dienoic PGs. Because the prostaglandins are synthesized from essential fatty acids which cannot be produced de novo in mammalian tissues—i.e., bis-homo-γ-linolenic (20:3ω6) and arachidonic (20:4ω6) acids—it seems reasonable that the dietary supply of linoleic acid can be an important modulating factor in PG biosynthesis.

The effects of diet manipulation on tissue fatty acid distribution and, ultimately, on PG production have been the object of several studies. These studies have shown that supplementation of a complete diet with ethyl arachidonate and ethyl bis-homo-γ-linolenate leads to their accumulation in all lipid classes (6), and that such enrichment of tissue lipids with these immediate PG precursors results in increased PG production in vivo (7,8). More recently, Nugteren et al. (9), Zöllner et al. (10) and Adam et al. (11,12) were able to establish a strong positive correlation between intake of the dietary PG precursor, linoleic acid, and the excretion rate of a tetranorprostanedioic acid (TNPDA), an analytical artifact which has been proposed as an index of the systemic turnover of primary prostaglandins of both the E and the F series (13). The variations of the intake levels considered in those latter studies, however, were rather drastic, typically 0, 10 and 50g linoleate/day (10). Consequently, the results cannot be considered a reliable guide to predict the effects of linoleate intake changes of the magnitude normally encountered in real life among and within human subjects over a period of time.

The primary objective of the present work, in which all the subjects were presumably in a PUFA nondeficient status before and during the study, was to determine if moderate changes in linoleate intake would modulate the systemic production of prostaglandins of the E series concurrently with, or independently of, changes in the plasma precursor levels. Indeed, published data suggest that PG biosynthesis is more directly related to dietary status than to the amount of PG precursors in the tissues (14-17). Thus, the secondary objective of our study was to demonstrate correlations, or lack of them, among dietary linoleate, plasma fatty acids, and PGE turnover as measured by the 24-hr urinary

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excretion of 11α-hydroxy-9,15-dioxo-2,3,4,5, 20-pentanor-19-carboxyprostanoic acid (PGE-M).

MATERIALS AND METHODS

Subjects

Four male volunteers 36, 37, 39 and 69 years of age weighing 83, 91, 70 and 93 kg, respectively, were recruited in the Beltsville, Maryland area. All four were in good health as determined by a complete physical examination and laboratory tests. None of the subjects took prescription medication, aspirin or aspirin-containing drugs during the study. All the procedures were approved by the Human Studies Committee of the U.S. Department of Agriculture and Georgetown University School of Medicine.

Controlled Diets

Two diets designated as L and H were formulated from food items commonly available to the American public. Both diets contained about 35% of energy (en%) as fat, 51-53% as carbohydrate and 13-14% as protein. The type of fat was varied between the two diets to obtain linoleic acid intakes of either 10 g/day (2.8 en%, diet L) or 30 g/day (8.4 en%, diet H). The major sources of linoleic acid in the diets were soft margarine, safflower, sunflower, soy and corn oils, and salad dressings prepared with the above oils. The menu cycle was 7 days; all diets were calculated to provide adequate amounts of nutrients known to be required by man, and for which data were available, as recommended by the Food and Nutrition Board of the National Academy of Sciences (1980 edition) at that time. Tables 1 and 2 summarize the nutrient content of diets in a typical 3200 kcal diet. All meals were prepared and weighed in our Human Diet Facility. Nutrient contents of the controlled diets were calculated from a data base consisting of the tape version of USDA Handbook 456 (18), the tape version of sections 1-7 of USDA Handbook 8 with supplementary items from sections 8 and 9, and material supplied by the food industry.

Experimental Design

The study was designed in four segments: period 1, a pre-study of three weeks in which the volunteers were free-living and eating a self-selected diet; period 2, lasting six weeks, in which the subjects were free-living but consuming the study diet (two of the subjects on each diet); period 3, a second 3-week self-selected diet period as in the pre-study, and period 4, six weeks free-living with a controlled diet (switchover from the diet in period 2). The controlled diet meals were eaten in the Human Diet Facility at the Beltsville Center on weekdays. Meals for Saturdays, Sundays and holidays were prepacked and distributed for home consumption. Caloric intake was adjusted where necessary by increasing or decreasing all foods so that the subjects maintained their weight throughout the study.

TABLE 1

Nutrient Content of a Typical 3200-Kcal Diet

Nutrient	Diet L	Diet H
Protein		
g/day	112	110
% of energy	14	13
Fat		
g/day	124	125
% of energy	34	35
Carbohydrate		
g/day	425	423
% of energy	53	52
Cholesterol (mg/day)	492	503

TABLE 2

Fatty Acid Content of a Typical 3200-Kcal Diet[a]

Fatty acid	Diet L	Diet H
Oleic (18:1ω9)		
g/day	49	45
% of energy	13.7	12.6
Linoleic (18:2ω6)		
g/day	10	30
% of energy	2.8	8.4
Total saturated		
g/day	49	36
% of energy	13.7	10.1

[a]Minor fatty acid constituents were: α-linolenic (18:3ω3), 1-2 g/day; arachidonic (20:4ω6), less than 0.3 g/day; eicosapentaenoic (20:5ω3), 0.02-0.1 g/day.

Sampling Procedures

Blood. Heparinized venous blood was drawn twice during periods 2 and 4 (controlled diet) and once during periods 1 and 3 (self-selected diet). The samples (whole blood) were centrifuged at 12,000 × g, and the resulting platelet poor plasma was analyzed for fatty acid content as indicated below.

Urine. Twenty-four-hr urine was collected on ice in glass bottles with 5 ml of toluene, six times during periods 1, 2 and 3, and nine times during period 4. After collection, the volumes were measured and aliquots were frozen in small

glass bottles at -20 C until assayed for the major metabolite of PGE_1 + PGE_2 (PGE-M) and creatinine.

Food composites. Three times during periods 1 and 3, 24-hr composites of duplicates of the food consumed were collected, homogenized, freeze-dried and analyzed for fatty acids as indicated below.

Analytical Methods

Blood. Plasma samples were extracted by a modified Sperry procedure (19). Total neutral lipids and phospholipids were separated by silicic acid column chromatography. Both fractions were then transmethylated and analyzed by gas-liquid chromatography according to a published procedure (19).

Food composites. The freeze-dried food composites were extracted with chloroform/methanol (2:1, v/v) according to a modification of the procedure of Folch et al. (20), and the fatty acid analysis was conducted in the same manner as for the plasma fatty acids.

Urine. The concentration of 11α-hydroxy-9, 15-dioxo-2,3,4,5,20-pentanor-19-carboxyprostanoic acid, the major metabolite of E prostaglandins (PGE-M), was determined in urine samples by combined gas chromatography-mass spectrometry according to a modification of a method fully described elsewhere (21). Briefly, a 20-ml portion of a 24-hr urine pool containing 1 μg of the diethyl ester of PGE-M as internal standard was acidified to pH 4.0 and extracted with Amberlite XAD-2 (Applied Science). After removal of the water phase by filtration, the resin was rinsed with 500 ml of water, then with 100 ml of light petroleum ether, and the organic materials were eluted with 120 ml of MeOH. The methanolic extract was rotoevaporated and the residue was methylated with CH_2N_2. The residue from solvent evaporation was dissolved in 0.5 ml of MeOH, then treated with 9.5 ml of H_2O/MeOH (85:10, v/v), and finally passed through a Sep-Pak cartridge (Waters Associates). The cartridge was then rinsed sequentially with 20 ml of water, 10 ml of petroleum ether and 5 ml of methyl formate/petroleum ether (5:95, v/v). The PGE-M, still bound to the octadecasilyl (ODS)-silica, was finally eluted with 10 ml of methyl formate/petroleum ether (25:75, v/v). The residue from solvent evaporation was dissolved in 50 μl of (water saturated) ethyl acetate/2,2,4-trimethylpentane (2:1, v/v), and chromatographed over a 1.5 g SiO_2 column. The first 6 ml of eluate was discarded. The next 12-ml fraction was collected and brought to dryness. The residue was treated with 30 μl of chloroform followed by 70 μl of *n*-heptane with stirring. A small precipitate that invariably formed was removed by centrifugation and the supernatant was chromatographed over Lipidex-5000 (Packard Instrument). The fraction containing PGE-M was evaporated and the residue was methoximated. Trimethylsilylation was conducted with 20 μl of N,O- bis(trimethylsilyl)trifluoroacetamide (Pierce). The sample was then ready for GC-MS analysis (21). The precision of the procedure, modified as described, was checked by analyzing four identical 20-ml urine specimens. The mean concentration (ng/20 ml) of PGE-M was 1042 ± 24 (SE) and the interassay coefficient of variation was 4.5%.

Creatinine was determined enzymatically with the Centrifichem (Union Carbide) automated system.

Statistics

Analysis of variance (SAS general linear model procedure) was used for statistical evaluation. Paired t tests (where differences between self-selected and high linoleate and self-selected and low linoleate intakes were analyzed) also were run. The results were similar to those obtained from the analysis of variance.

RESULTS AND DISCUSSION

The present study was designed primarily to explore systemic responses of PGE production to changes in dietary linoleate intake. We thought that the methodology of choice would be to measure the 24-hr urinary output of PGE-M, which is considered a reliable parameter for monitoring total body production of prostaglandins E_1 + E_2 (22). Consequently, we are neglecting the contribution of kidneys and seminal vesicles. The primary PGs synthesized by these organs are excreted, virtually unmetabolized, with the urine. Published data, however, indicate that the level of intrarenal primary PG biosynthesis (23,24) is at least two orders of magnitude smaller than the total body production (13,25-28). Therefore, in the context of our study, we can disregard their contribution. Conversely, to our knowledge, there are no published data on the level of prostaglandin biosynthesis by human seminal vesicles in vivo. Their contribution could possibly be a significant percentage of the total daily output. However, we have no way of assessing the impact of dietary manipulations on vesicular PGE production.

The results, summarized in Table 3, clearly demonstrate positive correlations between linoleate intake and linoleate concentration in the neutral lipid (P=0.007) and phosphoglyceride (P=0.012) fractions of plasma. Conversely,

TABLE 3

Plasma Linoleate, Plasma Arachidonate and Urinary PGE-M Responses to Changes in Linoleate Intakes

Subject	Period	Intake[a] g/day	Linoleate[b] Neutral lipid	Linoleate[b] Phospho-lipid	Arachidonate[b] Neutral lipid	Arachidonate[b] Phospho-lipid	PGE-M ng/g creatinine[c]
1	1	14.2	33.2	20.1	4.5	9.9	10.2 ± 1.3 (5)
	2	30.0	43.9	24.5	4.8	10.6	13.1 ± 1.2 (6)
	3	14.3	39.1	20.9	4.7	9.7	17.7 ± 2.7 (6)
	4	10.0	37.1	20.0	5.4	10.3	11.9 ± 0.8 (9)
2	1	13.3	39.9	20.3	5.2	13.4	10.7 ± 1.2 (6)
	2	10.0	33.3	19.6	5.5	12.5	10.0 ± 0.9 (6)
	3	18.1	40.3	23.4	5.6	12.5	15.0 ± 1.3 (6)
	4	30.0	47.4	22.5	6.8	13.5	15.4 ± 1.1 (9)
3	1	11.5	36.7	22.8	5.6	13.1	5.6 ± 0.8 (6)
	2	10.0	37.1	20.2	5.9	12.1	7.3 ± 0.4 (6)
	3	7.8	38.8	21.9	5.5	12.0	4.5 ± 1.1 (6)
	4	30.0	50.1	24.4	6.1	12.4	5.2 ± 0.4 (9)
4	1	14.1	42.0	20.6	6.4	13.2	26.1 ± 4.0 (6)
	2	30.0	49.9	23.5	7.2	12.6	28.1 ± 2.3 (6)
	3	12.9	44.2	21.0	8.6	15.4	27.2 ± 2.1 (6)
	4	10.0	39.5	20.1	6.9	12.5	29.7 ± 1.4 (9)

[a]During periods 1 and 3 the subjects ate a self-selected diet, and the linoleate intake was determined by analysis of composites of duplicates of the food consumed. During Periods 2 and 4 the subjects were fed a controlled diet, and the linoleate intake was calculated from *USDA food composition tables and information from the food industry (see text).*

[b]Plasma linoleate and arachidonate are expressed as % total fatty acids.

[c]Mean (± SE) values for PGE-M excretion (24-hr) are expressed as ng PGE-M/g creatinine (number of observations in parentheses).

neither plasma arachidonate concentrations nor the urinary PGE-M outputs of our subjects responded significantly to changes in linoleate intake. The lack of response of plasma arachidonate to dietary manipulations probably can be accounted for by the fact that 20:4ω6 levels in plasma, as well as in other tissues, are influenced by several biochemical processes, and by the fact that our subjects were already consuming sufficient amounts of PUFA. Dietary 20:4ω6, unlike 18:2ω6, does not contribute significantly to plasma 20:4ω6. Furthermore, neither α-linolenic (18:3ω3) nor eicosapentaenoic (20:5ω3) acids, at the levels of intake indicated in Table 2 (footnote), could have influenced the desaturation and elongation of linoleic acid.

Our data concerning the urinary PGE-M outputs would appear to conflict with results of previous studies where large responses in excretion of tetranorprostanedioic acid (TNPDA) (13) were observed concomitantly with changes in linoleate intakes (9-12). TNPDA represents the sum of the urinary levels of PGE-M, PGF-M and, conceivably of other C_{16} intermediate catabolites (29). There is no definitive proof as yet that interconversion between PGE and PGF takes place in the human in vivo through the agency of a prostaglandin 9-ketoreductase (E to F) and of a prostaglandin 9-hydroxydehydrogenase (F to E) (29). Recently, however, Rosenkranz et al. (30) observed a 4% conversion of (intravenously infused) PGE_1 to the metabolite of $PGF_{2\alpha}$. Mutual interconversion of PGE and PGF, if indeed it takes place to a considerable extent, clearly would be a complication factor that would require the measurement of both PGE-M and its counterpart, PGF-M (31), or of an aggregate parameter such as urinary tetranorprostanedioic acid (13).

Given the variety of factors that ultimately may determine the PGE, as well as the total PG, turnover, any tendency toward a higher level of PG production promoted by an increase in linoleate input from 10 to 30 g, and, conversely, any reduction induced by a reduced linoleate intake, might not be great enough to overcome all the endogenous factors that regulate PG production and excretion. This was probably true for the four volunteers who were selected for this study. The variations of 18:2ω6 intake considered in the experiments of Nugteren et al. (9), Zöllner et al. (10) and Adam et al. (11,12) were much larger than those considered in our

study. This one might have been the decisive factor that enabled those investigators to establish those strong positive correlations between linoleate intakes and excretion rates of TNPDA.

The present study was conceived and carried out with a view toward answering the question whether, in nondeficient states, amount and type of dietary fat could influence the activity of the PG system as measured by the daily PGE-M output. In spite of the limitation inherent in the choice of this chemical marker, positive evidence to that effect would significantly advance our understanding of the role of nutrition in the regulation of many physiological parameters. Our results do not necessarily contradict those of previous studies (9-12), partly because of limited comparability as discussed above. In view of our findings, however, and given the uncertainties and shortcomings noted above, one must consider the possibility that the urinary output of PGE-M alone is not a reliable indicator of total PG production. Thus, the original question that prompted us to conduct the study is still open. It is also worth emphasizing, however, that our observations and conclusions are valid only in subjects who already are consuming sufficient amounts of PUFA.

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The Effect of a Non-absorbable Fat on the Turnover of Plasma Cholesterol in the Rat

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ABSTRACT

Rats were injected with [4-^{14}C]-cholesterol and then fed diets that contained sucrose polyester (SPE) at levels of 0 and 8% of the diet. ^{14}C was measured in neutral and acidic steroid fractions of the feces collected during days 35-39 post i.v. injection. Periodic blood samples were used to measure the specific activity of the plasma cholesterol. The plasma data were consistent with a two-pool model for the decay of the plasma specific activity. The slow component of the decay curve decreased more rapidly in animals that received SPE. The half-life corresponding to this component was approximately 20% shorter in the SPE-fed animals compared to the control group. The mass of cholesterol calculated for the first pool was similar for all groups of animals. The ^{14}C found in the feces was consistent with the more rapid removal of cholesterol from the body in the SPE-fed animals. The mass of excreted steroid was equal to the calculated rate of cholesterol production in each group of animals.
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INTRODUCTION

Sucrose polyester (SPE) has physical properties similar to those of dietary triglycerides, but its physiological properties differ in that it is neither digested (1) nor absorbed (2-4). In the intestine, dietary cholesterol and fat-soluble vitamins distribute between this maintained oil phase of SPE and the micellar phase of bile salts, monoglycerides and free fatty acids (5,6). The absorption of these dietary lipophiles is lessened as a result of their retention in the oil phase of unabsorbable SPE (5-8). Besides the cholesterol in the diet, an even larger mass daily enters the intestinal lumen as a micellar phase in bile. A considerable portion of this cholesterol is reabsorbed in the ileum and the jejunum. Dietary SPE could reduce this absorption if the cholesterol in the biliary micellar phase transferred to the oil phase of SPE. The rate of transfer would need to be rapid because of the relatively short residence time of biliary cholesterol in the lumen of the intestine. Studies in vitro have shown that cholesterol transfers from a micellar phase, which simulates that found in the intestine, to an oil phase of SPE. Moreover, the rate of transfer was sufficiently rapid that it was likely that in the lumen of the intestine a significant amount of cholesterol would move from the micellar phase to the oil phase.

We report here the effect of dietary SPE on the reabsorption of biliary cholesterol, distinct from the previous reports of its effect on dietary cholesterol. Rats were injected with ^{14}C-cholesterol, and the effect of dietary SPE on the fall-off of the specific activity of plasma cholesterol was determined. If dietary SPE interfered with the reabsorption of biliary cholesterol, the decrease of the plasma ^{14}C-cholesterol specific activity would be accelerated. In addition, the feces of these animals were collected and analyzed for ^{14}C in the acidic and neutral steroid fractions. A decrease in the reabsorption of biliary cholesterol would be reflected in an increased rate of egestion of these ^{14}C-labeled steroids.

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MATERIALS AND METHODS

[4-^{14}C]-Cholesterol (New England Nuclear, Boston, Massachusetts) was found by TLC and assay of the scrapings from the plate to have a radiochemical purity of greater than 99%. SPE was synthesized by base-catalyzed esterification of sucrose with the fatty acids of safflower oil (18:2, 72%; 18:1, 16%; 16:0, 3%; 18:0, 3%) (8). The ester composition of the SPE as determined by TLC was 71% octaester, 25% heptaester and 4% hexaester. Palm oil was completely hydrogenated (HPO), and the fatty acid composition of the product was 46% palmitic acid and 51% stearic acid.

The compositions of the diets and their fat components are given in Table 1. The SPE was introduced into the 8-SPE diet as a replacement for an equal weight of soybean oil (SBO). When SPE was added to the diet (Add-SPE), all of the other components of the diet were reduced proportionally. Otherwise the diets were identical. All diets were free of cholesterol. HPO was added to the diets, by the replacement of soybean oil, to prevent anal leakage, which occurs

TABLE 1

A. Composition of Diet (wt. %)

Diet	All groups except Add-SPE	Add-SPE
Casein, vitamin free	27.0	25.0
Sucrose[a]	49.0	45.3
Fat (Table 1B)[b]	17.0	23.2
Salt Mix USP XVII[c]	4.0	3.7
Celluflour	3.0	2.8

[a]Water-soluble vitamins introduced by mixing with a portion of the sucrose as described previously (2).

[b]Fat soluble vitamins introduced by mixing with a portion of the soybean oil (2×10^4 IU retinyl palmitate, 2×10^3 IU D_3, and 100 IU D alpha tocopheryl acetate per kg diet).

[c]Salt mix was described previously (7).

TABLE 1

B. Composition of Fat Component (Wt. % of Diet)[a]

Group	SPE	HPO	SBO
SBO	0	0	17.0
SBO-HPO	0	2.0	15.0
8-SPE	8.0	2.0	7.0
Add-SPE	7.4	1.9	14.0

[a]SPE = Sucrose polyester; HPO = completely hydrogenated palm oil; SBO = soybean oil.

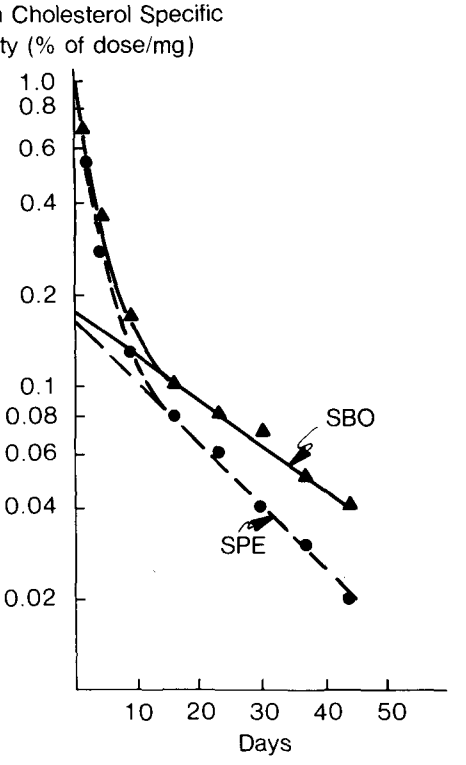

FIG. 1. Plasma cholesterol specific radioactivity (% of dose per mg cholesterol) decay curves of ^{14}C-cholesterol given intravenously at day zero. One rat (SBO) received a diet containing 17% soybean oil; the diet of the other rat (SPE) contained 7% soybean oil, 8% sucrose polyester and 2% completely hydrogenated palm oil.

on consuming diets containing high levels of SPE that is a liquid (9).

Four groups of 10 each young, adult male Sprague-Dawley rats were assembled by random selection. Throughout the study each group was fed ad libitum one of the experimental diets. After an initial seven days of adaptation to their diets, the rats were anesthetized with Nembutal. Each rat was injected in the penile vein with 1 ml of a suspension of ^{14}C-cholesterol (approximately 7 μg and 10 μCi/ml) in 1:16 ethanol/saline. The exact amount of ^{14}C-cholesterol that was injected was determined by weighing the syringe before and after the injection and by analysis of replicate samples of the suspension, which were taken at intervals during the time of the administrations.

On the second and fourth day and then at weekly intervals following the injection of ^{14}C-cholesterol, approximately 0.3 ml of blood was collected from each animal. The plasma was isolated and stored at 0 C until analyzed for total cholesterol (10) and for ^{14}C content by liquid scintillation counting.

The plasma cholesterol specific radioactivity decay curves of a rat that received the SBO diet and of a rat that received the 8-SPE diet are shown in Figure 1. These values, and those of the other rats, conformed to that of the two-pool model that has been reported for short term studies of cholesterol metabolism in man (11) and in the rat (12). The data points for each rat were fitted to a biexponential function by computer using the least squares technique. The values that were obtained for this function for each rat were used to calculate kinetic parameters of cholesterol turnover of a two-pool model (13). Since all animals were fed a cholesterol-free diet, de novo synthesis was the sole source of additional cholesterol over the course of the study. This model assumes further that the sole loss of cholesterol and its metabolites is from a single compartment of the body. The ultimate elimination of these compounds from the body is chiefly as a component of the feces.

Feces were collected on days 35 to 39 post-injection. These samples were stored at 0 C until analyzed. The neutral and acidic steroids of the

TABLE 2

Group Mean Plasma Cholesterol Turnover Values in Rats Consuming Diets Containing Triglycerides or Triglycerides and Sucrose Polyester*

	SBO		SBO-HPO		8-SPE		Add-SPE	
Symbols & units**	Mean	SE	Mean	SE	Mean	SE	Mean	SE
$t_{1/2\alpha}$, day	1.87	0.09	1.74	0.08	1.86	0.32	1.46	0.09
$t_{1/2\beta}$, day	19.9[a]	0.9	16.9[a]	0.5	16.6[b]	0.9	14.2[c]	0.05
M_A, mg	89.6	5.5	87.3	4.8	90.6	8.0	82.7	7.3
PR_A, mg/day	14.1[a]	0.5	15.1[a]	0.4	17.2[b]	0.6	17.6[b]	0.9
k_A, day^{-1}	0.160[a]	0.008	0.177[a,b]	0.010	0.199[b,c]	0.013	0.224[c]	0.016
$-k_{AA}$, day^{-1}	0.332[a]	0.020	0.356[a,b]	0.023	0.377[a,b]	0.034	0.428[b]	0.028
k_{AB}, day^{-1}	0.171	0.013	0.179	0.014	0.178	0.022	0.205	0.013
k_{BA}, day^{-1}	0.088[a]	0.004	0.096[a,b]	0.003	0.091[a]	0.007	0.109[b]	0.005

*SE = standard error, n = 10 per group. Values in the same row with different superscript letters differ significantly ($P \leq 0.05$).

**$t_{1/2\alpha}$, half-life of first exponential. $t_{1/2\beta}$, half-life of second exponential. M_A, size of pool A. PR_A, production rate in pool A. k_A, rate of irreversible elimination from pool A and the whole system. k_{AA}, rate of removal from pool A by all routes. k_{AB}, rate of transfer from pool A to pool B. k_{BA}, rate of transfer from pool B to pool A.

stools were obtained by the method of Grundy et al. (14). The ^{14}C content of aliquots of the aqueous (bile acid) and hexane (neutral steroid) phases was determined by scintillation counting.

Initial statistical treatment of the data was by one-way analysis of variance. If a treatment effect was determined to be significant by this method ($P<0.05$), the data were then examined by t-test (15).

RESULTS

Over the course of the study the mean gain in body weight by all groups of rats was essentially the same. As reported before (7), the unabsorbability of SPE and HPO was reflected in an increase in food consumption in proportion to the level of these two dietary components. This increase resulted in the absorbable energy consumed by all groups being approximately the same, about 95 kcal/day.

The specific activity of the plasma cholesterol was determined. The mean values of the kinetics of cholesterol turnover for each of the dietary groups are given in Table 2. Over the six weeks of measurements following the injection of ^{14}C-cholesterol, the total plasma cholesterol of the group receiving the SBO-HPO diet increased approximately 15%. The level of this plasma component in the other groups remained essentially constant throughout the study at approximately 100 mg/dl. The small but statistically significant increase in the total plasma cholesterol level of the rats that received the SBO-HPO makes their values unusable for this type of kinetic analysis (12). Consequently, the values calculated for that group are not considered in the following comparisons.

Relative to the group of rats that received the SBO diet, the ingestion of SPE resulted in several significant changes in cholesterol metabolism. The halflife of the elimination phase, $t_{1/2}$, was reduced from 19.9 days (SBO group) to 16.6 days (8-SPE group) and 14.2 days (Add-SPE group). This reduction was reflected in an approximate 25% increase in the elimination rate, k_A, by the two groups that received SPE. Accompanying this increased elimination was an equivalent, 25% increase in the production rate, PR_A. Because of these offsetting changes in elimination and syntheses, the mass of cholesterol in the first pool, M_A, was unaltered.

During days 35 to 39 post-injection, the percent of the administered ^{14}C-cholesterol that was excreted in the feces as neutral and acidic steroids was determined. The percentage values for ^{14}C egestion were converted into absolute values of cholesterol mass by using the specific activity of the plasma cholesterol. In that calculation, allowance must be made for the time of movement of labeled steroids from the plasma pool to the feces. Zilversmit and Hughes (16) have shown the transfer time for cholesterol from the plasma to the neutral steroid fraction of the feces to be 2 days. Since the midpoint of the 5 days of feces collection was day 37, day 35 was the corresponding time when the cholesterol that would appear in the feces as neutral steroids left the plasma pool. The mass of neutral steroid (M_n) excreted daily in the feces was calculated from the equation $M_n = C_n/C_{35}$,

where C_n is the mean percentage of the dose egested daily as neutral steroids on days 35 to 39 and C_{35} is the plasma specific activity on day 35. The mass of bile acids that was egested in the feces was calculated in a similar manner. Cohen et al. (17) have shown the time of transfer of labeled steroid from the plasma to appearance as fecal acidic steroids to be 6 days. Thus, for the acidic steroids in the feces of midpoint day 37, day 31 would be the corresponding day when the steroids from which they were derived left the plasma pool. The mass of fecal acidic steroids was calculated from the equation $M_a = C_a/C_{31}$, where C_a is the mean percentage of the isotope egested daily as acidic steroids on days 35 to 39, and C_{31} is the plasma specific activity on day 31.

The mean daily egestion of neutral and acidic steroids by the four dietary groups that were studied is shown in Table 3. The amount of neutral steroids in the feces of the rats that consumed diets containing SPE (8-SPE and Add-SPE groups) was approximately twice that of the rats that received the diet containing soybean oil (SBO group). There were small, but statistically significant, increases in acidic steroid egestion in the SBO-HPO and Add-SPE groups.

DISCUSSION

As shown in Table 2, the consumption of diets containing SPE resulted in an increased production (PR_A) of cholesterol but without an increase in pool size (M_A). Since k_{AB} was unchanged, the maintenance of pool size was not a consequence of increased transfer between pools. Rather, the increased production was proportional to the increased rate (k_A) of loss in the feces. This relationship also was shown by the mass of steroid that was egested daily (Table 3). The total amount found in the feces of each of the dietary groups was quite similar to the daily production rates reported in Table 2. The correlation between the individual animal's production rates and fecal steroid excretion was significant ($R = 0.60$, $P<0.0001$). The increased turnover of plasma cholesterol that accompanies the ingestion of SPE is then a consequence of the decreased absorption of enterohepatic circulating steroids, mainly cholesterol, with an ensuing increased synthesis of cholesterol. The amount of synthesized cholesterol is equal to that lost in the feces and as a consequence, the total mass of body cholesterol is unchanged.

TABLE 3

Mean Daily Fecal Excretion of Neutral and Acidic Steroids. Calculated from Fecal Excretion of ^{14}C on Days 35 to 39 and from Plasma Specific Activity*,**

Group	Mg per day ± standard error		
	Acidic	Neutral	Total
SBO	9.5 ± 0.4[a]	3.9 ± 0.3[a]	13.5 ± 0.6[a]
SBO-HPO	11.0 ± 0.5[b]	4.9 ± 0.2[b]	15.9 ± 0.7[b]
8-SPE	11.1 ± 1.0[a,b]	6.7 ± 0.3[c]	17.8 ± 1.3[b]
Add-SPE	10.9 ± 0.5[b]	7.9 ± 0.4[d]	18.8 ± 2.5[a,b]

*Within each column the values with different superscripts are significantly different ($P \leq 0.05$). n = 10/group.

**See text for a description of the method of calculation.

The increased loss of steroids in the feces of the animals fed SPE can be related to the physical and physiological properties of this lipid. SPE is similar to triglycerides in its ability to dissolve cholesterol. They are alike also in having an oil/water distribution coefficient of 135 where the aqueous phase simulates that found in intestinal lumen contents (6). They differ in that SPE is not digested and as a consequence is not absorbed. SPE and the cholesterol that is dissolved in it are eliminated together in the feces. As we have shown earlier (5), there is a resulting decrease in the absorption of dietary cholesterol. The study reported here shows a similar effect on enterohepatic circulating cholesterol. The minimal effect of SPE on the excretion of bile acids observed in this study, and as reported previously (6), is consistent with their lack of affinity for the SPE oil phase.

That dietary SPE can decrease the reabsorption of enterohepatic circulating cholesterol has been indicated in studies with human subjects (9). In those studies, the subjects received diets that supplied approximately 800, 300 or less than 50 mg of cholesterol per day. The introduction of SPE into these diets resulted in a decrease in plasma cholesterol at all levels of cholesterol intake. A portion of this decrease was a likely consequence of a decreased absorption of dietary cholesterol. The reduction in plasma cholesterol that was seen at the lowest level of cholesterol intake was interpreted as pointing to a decreased absorption of enterohepatic circulating cholesterol. The studies reported here show that this explanation is probably true.

The procedure we used here provides a method by which the mass of excreted steroids can be determined from the amount of ^{14}C in the feces and the specific activity of the plasma cholesterol; plasma die-away data are not necessary. Thus, the animal would be injected with ^{14}C-cholesterol and the feces subsequently collected over a period of a few days. The specific activity of the plasma cholesterol would be determined two and six days prior to the

midpoint of the fecal collection period and the percent of the administered ^{14}C in the fecal neutral and acidic steroids would be determined. Calculation of the mass of these two steroid classes would be as described in the Results section. The period during which these collections are made should be after the contribution of the first component of the plasma die-away curve is insignificant. As shown in Figure 1, the collection period should be after day 21.

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Unsaturated Fatty Acid Compositional Changes and Desaturation During the Embryonic Development of the Chicken (*Gallus domesticus*)

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ABSTRACT

An investigation has been made to correlate the activities of the Δ9- and Δ6-long chain fatty acid desaturation systems with the increased levels of oleic and arachidonic acids in the liver relative to the yolk sac membrane of the chick embryo during the last week of development. The membrane exhibited high levels of both stearic and linoleic acid desaturation in the early stages of yolk lipid mobilization, the activities of both enzyme systems decreasing with the approach of hatching. Stearic acid desaturation in the liver also decreased with the approach of hatching, but linoleic acid desaturation increased. The observed levels of desaturation in the yolk sac membrane are capable of making a considerable contribution to the accumulations of mono- and polyunsaturated fatty acids in the embryonic liver, the requirement for which does not appear to be satisfied by the yolk lipids. With the approach of hatching and the functional regression of the yolk sac membrane, the role is taken over by the embryonic tissues.

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INTRODUCTION

Virtually the entire lipid content (5-6 g) of the yolk consisting in the main of triglycerides (72% of the total lipids) and phospholipids (22% of the total lipids) is metabolized and absorbed into the chick embryo during the last seven days of its 21-day period of incubation (1,2). Many unique features in lipid metabolism, especially within the liver, accompany this rapid assimilation of lipid (2-6). Two of the features of the hepatic lipid metabolism are (i) an extensive accumulation of lipid which is predominantly accounted for by cholesteryl oleate such that by the 19th day of incubation cholesteryl oleate accounts for >65% of the total hepatic lipid, and (ii) the phospholipids, which comprise some 10% of the total lipid present, contain unusually high levels (>20%) of arachidonic acid, particularly during the early part of yolk lipid mobilization. These observations cannot be explained by the composition of the yolk lipids. For instance, within the yolk contents cholesteryl oleate accounts for <0.5% of the total lipids present (7) while arachidonic acid comprises only 3% of the total fatty acids in the phospholipids. On the other hand, the yolk sac membrane, which undergoes considerable development during the last week of incubation and through which all the yolk lipid has to pass before assimilation (8), does possess both fatty acid compositional and metabolic features which indicate that it plays an important role in the hepatic accumulation of the cholesteryl oleate and the highly unsaturated phospholipids (7,9). The accumulation of such large quantities of oleic and arachidonic acids in the liver of the chick embryo has, therefore, prompted an investigation into the relative activities of the Δ9- and Δ6-long chain fatty acid desaturation systems in the yolk sac membrane and liver during the last week of incubation.

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EXPERIMENTAL PROCEDURES

Animals and Tissues

Fertile eggs were obtained from a commercial flock of 9-mo old Ross 1 broiler-breeder parent stock which were kept on deep litter and received a proprietary diet formulated for breeding birds. Embryos were taken from incubated eggs at 15, 17 and 19 days and the yolk sac membrane and liver excised as described previously (2,7). The tissues were chilled immediately in ice-cold homogenization buffer consisting of 320 mM sucrose and 1 mM EDTA in 7.5 mM sodium phosphate buffer, pH 7.4. 10 g of the tissues were chopped roughly with scissors and homogenized in a laboratory emulsifier (Silverson Machines Ltd., Chesham, England) in 25 ml of ice-cold sucrose-EDTA-phosphate buffer. Any residual connective tissue was removed by filtration through a 2 mm gauge stainless steel wire gauze. To derive sufficient material it was necessary to pool the livers obtained from 32 embryos for each observation. Observations on the yolk sac membrane were conducted using pooled material from 8 embryos.

Assay Procedure for Desaturation Activities

1 ml of the yolk sac membrane or liver homogenate was added to 1 ml of the following assay mixture: ATP (3.5 mM), coenzyme A (0.1 mM), NADH (0.5 mM), NADPH (0.5 mM), acetyl coenzyme A (0.1 mM), malonyl coenzyme A (0.1 mM), $MgCl_2$ (2.4 mM) and [1-^{14}C]-stearic or linoleic acids (5 μM:0.5 μCi) complexed with bovine serum albumin (10). Following incubation at 37 C for 2 hr, the lipids were extracted from the tissue homogenates with chloroform:methanol (2:1, v/v) (11) in the presence of 100 g/ml of 2,6-di-tert-butyl-p-cresol as an antioxidant. The major lipid components were separated on thin layers of silica gel G (E. Merck, Darmstadt, Germany) by development in hexane:ether:formic acid (80:20:1, v/v/v). The major neutral lipid fractions consisting of cholesteryl esters, triglycerides, unesterified fatty acids and partial glycerides were eluted from the silicic acid by washing with diethyl ether and the phospholipids by washing with acidified methanol. The bands of esterified lipid were pooled and an aliquot of the total esterified lipids and the unesterified fatty acids was dried down and, following dissolution in a toluene-based scintillant, the radioactivity was determined using a liquid scintillation counter (Packard 2425, Packard Instruments Ltd., Downers Grove, Illinois, U.S.A.). The remainder of the esterified lipids and unesterified fatty acids were transmethylated by refluxing in methanolic HCl (12) and the methyl fatty esters separated according to their degree of unsaturation by argentation chromatography (13). Bands corresponding to saturated, monoenoic, dienoic, trienoic and tetraenoic fatty acids were eluted with diethyl ether and, following removal of the solvent, their radioactivity was determined as described above. Confirmation of the fatty acid grouping was obtained by co-chromatographic separation of standard methyl esters and subsequent gas chromatographic identification. Quantification of the relative proportions of each of the major lipid bands and their long chain fatty acid compositions was performed on samples of unincubated tissues by a combination of a densitometric charring procedure following separation on thin layer chromatoplates (14) and transmethylation in the presence of a heptadecanoic acid standard followed by gas liquid chromatography. The degree of dilution of the ^{14}C fatty acid substrates by their respective tissue pools was determined from the pool size of unesterified stearic and linoleic acids, thereby enabling the desaturase activities to be calculated in terms of pmoles of fatty acid metabolized.

TABLE 1

Lipid Contents and Compositions (Weight Percentages of Total Lipid) of the Yolk Sac Membrane and Livers of the Chick Embryos

Stage of development (days)	15			17			19		
Yolk sac membrane: g Lipid (total per membrane)		0.58			2.06			1.12	
	CE	TG	PL	CE	TG	PL	CE	TG	PL
Composition[a]	10.9 ±1.27	64.8 ±2.23	16.9 ±0.72	9.33 ±0.59	69.9 ±2.02	14.2 ±1.47	7.88 ±1.20	73.4 ±3.22	11.1 ±1.44
Liver: mg Lipid (total per liver)		20.8			50.0			95.6	
	CE	TG	PL	CE	TG	PL	CE	TG	PL
Composition[b]	35.1 ±2.11	15.1 ±1.49	35.8 ±3.56	41.6 ±2.09	18.9 ±4.00	27.1 ±1.39	62.8 ±0.53	8.88 ±1.19	18.0 ±1.06

CE = cholesteryl ester; TG = triglyceride; PL = phospholipid.

Each result is the mean ± S.E. of 6 observations.

[a]Free cholesterol accounted for 4-5%, unesterified fatty acids 2-3% and partial glycerides <1.0% of total lipid.

[b]Free cholesterol accounted for 8-9%, unesterified fatty acids 2-3% and partial glycerides <1.0% of total lipid.

Assay of the Protein Content

Protein was assayed by the method of Lowry et al. (15) as modified by Miller (16). The intensity of the blue color produced was measured by means of a liquid scintillation counter as described by Noble and Shand (17).

RESULTS

The total lipid contents and relative concentrations (weight percentages of the total lipid) of the cholesteryl esters, triglycerides and phospholipids, i.e. the major fatty acid-containing lipid fractions, in the yolk sac membrane and liver of the chick embryo at days 15, 17 and 19 of incubation are given in Table 1. Transfer of lipid from the yolk contents during this period of incubation was associated with considerable changes in the total lipid content of the yolk sac membrane. The large increase in total lipid content of the yolk sac membrane which occurred between days 15 and 17 of incubation was followed by a decline between days 17 and 19. Triglycerides accounted for more than half of the lipid accumulation and were accompanied by appreciable amounts of both phospholipids and cholesteryl esters. Between days 15 and 19 of incubation the total lipid content of the liver increased nearly 5-fold. Although by far the largest proportion (some 63%) was accounted for by the accumulation of cholesteryl esters, there also was a 2-3-fold increase in the overall amount of phospholipids.

The relative concentrations (weight percentages of the total fatty acids present) of stearic, oleic, linoleic and arachidonic acids within the three major fatty acid-containing lipid fractions of the yolk sac membrane and livers of the chick embryo at days 15, 17 and 19 of incubation are shown in Table 2. There were no appreciable changes in the relative distributions of the fatty acids within the yolk sac membrane over the period of the incubation. In all the fractions, oleic acid was the major fatty acid present, its level being particularly high in the cholesteryl esters. Although linoleic acid was present in all the fractions, in the phospholipids it was accompanied by high levels of arachidonic acid. In the liver, also, there were no appreciable changes in the relative distributions of the fatty acids between days 15 and 19 of incubation. The relative distributions of the

TABLE 2

Proportions of Stearic, Oleic, Linoleic and Arachidonic Acids (Weight Percentages of the Total Fatty Acids Present) in the Cholesteryl Ester, Triglyceride and Phospholipid Fractions of the Yolk Sac Membranes and Livers of the Chick Embryo

	Cholesterol ester			Triglyceride			Phospholipid		
Stage of development (days)	15	17	19	15	17	19	15	17	19
Yolk sac membrane:									
Stearic	5.35 ±0.90	6.71 ±0.39	6.76 ±1.69	7.07 ±0.36	6.43 ±0.20	6.56 ±0.62	17.8 ±0.72	17.9 ±0.99	20.0 ±0.56
Oleic	71.4 ±2.97	70.8 ±4.99	73.0 ±5.86	53.1 ±1.32	55.7 ±1.45	50.7 ±2.44	32.9 ±0.71	36.5 ±2.31	32.2 ±3.89
Linoleic	10.3 ±2.29	7.00 ±1.26	9.76 ±1.44	10.1 ±1.50	8.70 ±0.54	15.2 ±3.93	12.1 ±0.71	10.4 ±0.56	16.2 ±3.33
Arachidonic	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	7.5 ±1.13	5.75 ±0.37	7.83 ±1.26
Liver:									
Stearic	4.00 ±0.39	4.12 ±0.38	3.38 ±0.15	16.0 ±1.84	11.0 ±0.54	12.5 ±0.20	25.0 ±0.42	24.0 ±0.77	26.8 ±0.69
Oleic	78.2 ±1.50	81.5 ±0.74	78.9 ±2.08	42.5 ±1.79	50.2 ±0.19	49.4 ±2.89	11.0 ±0.61	14.7 ±0.72	11.4 ±0.08
Linoleic	7.88 ±0.84	7.86 ±0.35	12.9 ±1.28	8.76 ±1.05	9.11 ±0.45	11.1 ±1.83	13.0 ±0.68	14.0 ±0.52	17.2 ±0.60
Arachidonic	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	27.5 ±1.23	25.7 ±0.22	26.9 ±1.27

Each result is the mean ± S.E. of 6 observations.

TABLE 3

The Δ9- and Δ6-desaturation Activities (pmoles of Acid Desaturation/2 hr/mg Protein × 10^{-2}) of the Yolk Sac Membranes and Livers of the Chick Embryos

Stage of development (days)	15	17	19
Yolk sac membrane:			
Stearic acid desaturation	39.5 ±11.2	31.4 ±1.59	17.2 ±0.48
Linoleic acid desaturation	28.1 ±4.93	6.11 ±0.88	7.92 ±1.32
Liver:			
Stearic acid desaturation	61.7 ±6.88	19.3 ±2.95	20.9 ±4.08
Linoleic acid desaturation	11.1 ±0.96	13.9 ±0.79	15.7 ±1.68

fatty acids within the cholesteryl ester and triglyceride fractions were very similar to those in the yolk sac membrane, with the cholesteryl esters containing a particularly high level of oleic acid. In contrast, in the phospholipid fraction of the liver, arachidonic acid accounted for over 25% of the total fatty acids present, that is, 4 times as much as in the phospholipid fraction of the yolk sac membrane.

Table 3 shows the desaturation of stearic acid (pmoles stearic acid converted to oleic acid per mg protein) and linoleic acid (pmoles linoleic acid converted to arachidonic acid per mg protein) in the yolk sac membrane and livers of the chick embryos during the 2-hr in vitro incubations. The range of desaturation activities within the yolk sac membrane and liver were of a similar order. The yolk sac membrane displayed high levels of both Δ9- and Δ6-desaturation at day 15 of incubation, i.e., the earlier stages of yolk lipid mobilization; at day 19 of incubation, i.e. with the approach of hatching, the activities of both desaturation systems had decreased markedly. The liver also displayed a high level of the Δ9-desaturation at day 15 of incubation, which decreased significantly by day 19 of incubation. However, in contrast to the yolk sac membrane the activity of the Δ6-desaturation in the liver increased between the 15th and 19th days of incubation.

DISCUSSION

Extensive qualitative and quantitative changes occur among the major lipid fractions within the various tissue pools of the chick embryo during the last week of development (2,4,7). The absorption of the large store of lipid within the yolk contents, consisting in the main of triglycerides and phospholipids, occurs through the mediation of a well-developed and highly vascularized yolk sac membrane (8) whose concentration of lipid increases considerably during this period. The overall similarity in comparative lipid and fatty acid compositions of the yolk contents and yolk sac membrane precludes any extensive degradation and resynthesis of the triglycerides and phospholipids during their absorption from the yolk contents into the membrane (7). Whereas it also has been proposed that the triglycerides are transferred into the embryo without substantial hydrolysis (18), there is evidence that extensive degradation and resynthesis of the phospholipids occurs (2,18-21).

Previous investigations have demonstrated the influence of the yolk sac membrane upon the lipid composition of the embryonic tissues and liver in particular (5,7,9). Further evidence of this relationship now is provided by the observation of appreciable Δ9- and Δ6-desaturation within the yolk sac membrane and liver during the period of intense yolk lipid absorption. Recent results (5,9) have confirmed that the yolk sac membrane, which is the major site of lipoprotein synthesis and assembly of the absorbed yolk lipids, is also the major site of synthesis for the cholesteryl oleate which subsequently accumulates to such a large extent in the liver. The importance of the cholesteryl oleate for the stability of the very low density lipoprotein complexes involved in the embryonic lipid transport has been suggested (5, 9). The source of the oleic acid for the cholesteryl ester synthesis in the yolk sac membrane is unknown. Although there is no shortage of oleic acid available from the triglycerides and phospholipids that accumulate in the yolk sac membrane during their passage into the embryo, there is no evidence of any preferential

hydrolytic release of oleic acid for esterification with cholesterol. The presence of high levels of Δ9-desaturation in the yolk sac membrane during the most active period of yolk lipid absorption, i.e. days 15 to 19 of incubation, suggests a possible mechanism whereby a considerable contribution may be made to the oleic acid requirement of the cholesterol esterification process.

The accumulation in the liver of phospholipids containing levels of arachidonic acid which greatly exceed the levels present in the phospholipids of the yolk sac membrane and contents is indicative of active desaturation and chain elongation of linoleic acid. Evidence already has been presented for the role of the yolk sac membrane in the differential absorption of phosphatidylethanolamine species from the yolk contents, in particular those phosphatidylethanolamine species containing high levels of polyunsaturated fatty acids (2,7,21,22). As a result, levels of docosahexaenoic acid (22:6[n-3]) well above those found in the yolk contents have been found in the yolk sac membrane, liver and other tissues of the embryo. Although the yolk sac membrane also was shown to contain levels of arachidonic acid in the phospholipids that were well above those of the yolk contents, no preferential absorption of arachidonic acid containing phospholipid species from the yolk contents was observed. As a large proportion of the phospholipids of the chick embryo arises through re-synthesis (2, 18-21), the presence of extensive Δ6-desaturation activity within the tissues, but particularly within the yolk sac membrane during the earlier stages of yolk lipid absorption, presents an opportunity to enhance the arachidonic acid level of the absorbed material. The considerably higher levels of arachidonic acid found in the phospholipids of the liver reflect the rapid mobilization of the arachidonic acid following synthesis (22).

Notable decreases have been observed in the metabolism and lipid compositional changes associated with the yolk sac membrane during the last few days before hatching (7,9) and are indicative of its functional regression. The role of the yolk sac membrane in the synthesis of unsaturated fatty acids is no exception to this and presumably is geared to a high requirement during embryonic cell growth for a range of fatty acids containing in particular arachidonic acid, which cannot be adequately satisfied by the more saturated fatty acids of the yolk lipids. In this respect there is much similarity with synthesis of arachidonic acid by the placenta during the embryonic development of various mammals (23). With the functional regression of the yolk sac membrane on the approach of hatching, the liver becomes far more important in the provision of arachidonic acid.

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Influence of Dietary Partially Hydrogenated Vegetable and Marine Oils on Membrane Composition and Function of Liver Microsomes and Platelets in the Rat

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ABSTRACT

The aim of the present study was to investigate the influence of partially hydrogenated vegetable and marine oils on membrane composition and function of liver microsomes and platelets with particular reference to the metabolism of linoleic acid and the production of arachidonic acid metabolites. Four groups of male weanling rats were fed linoleic acid supplemented diets containing 20% (w/w) of partially hydrogenated low erucic acid rapeseed oil (HLRSO), partially hydrogenated herring oil (HHO), olive oil (OO) and trierucin + triolein (TE) for 10 weeks. An additional two groups were fed partially hydrogenated low erucic acid rapeseed oil and partially hydrogenated herring oil without linoleic acid supplementation (HLRSO- and HHO-, respectively).

Substantial amounts of *trans* fatty acids were incorporated into liver microsomes (12.6% in group HLRSO) and platelets (7.0% in group HLRSO-). This incorporation was not dependent on the dietary linoleic acid level. Hepatic microsomal Δ^5-desaturase activity was significantly increased after HLRSO feeding compared to OO feeding. Δ^6-Desaturase activity did not vary in the linoleic acid supplemented groups. Both Δ^5- and Δ^6-desaturase activities were significantly increased in groups without linoleic acid supplementation.

Docosenoic acid was incorporated into platelet phospholipids in contrast to liver microsomes. In the platelet, docosenoic acid seemed to have a special preference for phosphatidylserine. Very small amounts were incorporated into platelet phosphatidylinositol. Feeding diets HLRSO, HHO and OO did not influence rat platelet cyclooxygenase or 12-lipoxygenase activity. Platelets from rats fed TE, however, produced significantly less 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) than platelets from rats fed OO. Feeding of HLRSO- and HHO- resulted in a significantly diminished production of the arachidonic acid metabolites 12-HETE, 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and 6-keto-prostaglandin $F_{1\alpha}$ in stimulated platelets and aorta. Thus, high dietary levels of *trans* isomers of monoenoic acids do not interfere with platelet cyclooxygenase or lipoxygenase activity provided sufficient amounts of linoleic acid are available.

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INTRODUCTION

In previous studies the influence of dietary partially hydrogenated vegetable and marine oils as well as unhydrogenated rapeseed oil on rat heart mitochondrial membrane fatty acid composition was studied with particular reference to cardiolipin and oxidative phosphorylation (1,2). In the present investigation, the influence of dietary partially hydrogenated vegetable and marine oils on membrane composition and function of liver microsomes and platelets was studied with emphasis on the metabolism of linoleic acid and the production of arachidonic acid metabolites.

Our knowledge of the biochemical and metabolic effects of geometrical and positional isomers of dietary fatty acids on the metabolism of essential fatty acids and the product-precursor relationship between essential fatty acids and oxygenated fatty acid metabolites is incomplete. Partially hydrogenated vegetable oils, which are consumed in substantial amounts, contain a wide range of positional isomers mainly of monounsaturated fatty acids with the double bond in the *trans* as well as in the *cis* configuration (3). Animal studies have shown that dietary *trans* octadecenoic acids have the ability to exaggerate the signs and symptoms of essential fatty acid deficiency (4,5). Positional and geometrical isomers of octadecenoic acid have been reported to inhibit liver microsomal desaturation of linoleic acid and dihomogamma linolenic acid in vitro (6,7). The inhibitory effect of dietary isomeric octadecenoic acids on the conversion of linoleic acid to arachidonic acid may in part explain the aggravation of symptoms of essential fatty acid deficiency after feeding partially hydrogenated oils. In some recent reports from this laboratory (1,8), we have studied the effects of dietary partially hydrogenated marine and vegetable oils on mitochondrial function and membrane fatty acid composition as well as on Δ^5- and Δ^6-desaturase activities. In these studies it was demonstrated that isomers of docosenoic

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acid from vegetable and marine oils have the ability to interfere with cardiac ATP synthesis and to alter the fatty acid composition of the cardiolipin of rat heart mitochondria. These studies also demonstrated that a dietary partially hydrogenated marine oil affected the metabolism of linoleic acid in rat liver microsomes.

The release of arachidonic acid from phospholipids can occur through two major pathways, one by the direct action of phospholipase A_2, liberating free acid, and the other via phospholipase C and a diglyceride lipase (9, 10,11). Depending on the fatty acid composition of the phospholipids acting as substrates for the different enzymes, the two pathways can contribute to a different extent to the release of arachidonic acid. It is apparent that a complete characterization of the phospholipid and fatty acid composition of platelets after dietary manipulation is useful for understanding the relative importance of the dietary fatty acids for the production of oxygenated arachidonic acid metabolites.

In the present investigation we have extended our earlier studies on the incorporation of dietary isomeric fatty acids into membrane phospholipids to liver microsomes and platelets after feeding partially hydrogenated marine and vegetable oils to rats. In particular, we have studied the influence of dietary long-chain monoenoic fatty acids on the composition of platelet phospholipids and on the production of arachidonic acid metabolites via the cyclooxygenase and 12-lipoxygenase pathways. In addition, we have investigated the dietary influence of isomeric long-chain fatty acids on the fatty acid composition of liver microsomes and microsomal Δ^5- and Δ^6-desaturase activities. In the present study we have used diets with and without linoleic acid supplementation to assess the influence of the dietary linoleic acid level on the incorporation of isomeric fatty acids into membranes of different tissues.

TABLE 1

Composition of Experimental Diets with Regard to Oil Content

Diet	Oil	Weight-%
HLRSO	Partially hydrogenated low erucic acid rapeseed oil	16.5
	Sunflowerseed oil	3.5
HHO	Partially hydrogenated herring oil	16.5
	Sunflowerseed oil	3.5
OO	Olive oil	18.0
	Sunflowerseed oil	2.0
TE	Triolein	13.0
	Trierucin	5.0
	Sunflowerseed oil	2.0
HLRSO-	Partially hydrogenated low erucic acid rapeseed oil	20.0
HHO-	Partially hydrogenated herring oil	16.1

MATERIALS

Partially hydrogenated low erucic acid rapeseed oil (Lobra 40 C), partially hydrogenated herring oil, sunflower seed oil, olive oil and trierucin were supplied by Dr. R. Ohlson, Karlshamns Oljefabriker, Karlshamn, Sweden. DL-α-Tocopherol was obtained from Sigma Chemical Co. (St. Louis, Missouri). Methanolic sodium methoxide (0.5 M) was obtained from Applied Science Europe B.V. (Oud-Beijerland, The Netherlands). Methanolic hydrogen chloride (1 M) was prepared from hydrogen chloride, purity 99% (AGA Special Gas, Lidingö, Sweden). Thrombin (Topostasin®) was obtained from F. Hoffman-La Roche & Co. AG (Basel, Switzerland). HPLC solvents were purchased from Rathburn Chemical Ltd. (Walkerburn, Scotland). [1-^{14}C]linoleic acid (sp. act. 56 mCi/mmole) and [1-^{14}C] eicosatrienoic acid (sp. act. 55 mCi/mmol) were purchased from The Radiochemical Centre (Amersham, Great Britain) and were 99.8% and 99.2% radiochemically pure, respectively, as judged by HPLC with radioactivity detection. Linoleic acid, γ-linolenic acid, eicosatrienoic acid and arachidonic acid were purchased from Nu-Chek-Prep (Elysian, Minnesota). Phospholipid standards were purchased from Supelco Inc. (Bellefonte, Pennsylvania). 13-Hydroxy-6,9,11-octadecatrienoic acid was a gift from Dr. M. Hamberg (Karolinska Institutet, Stockholm, Sweden). All other reagents and chemicals were purchased from Merck (Darmstadt, German Federal Republic).

EXPERIMENTAL DESIGN

Animals

Male weanling Sprague-Dawley rats, divided into six groups of 10 rats each, were fed semisynthetic diets containing 20% (w/w) of different fats for a period of 10 weeks. The composition of the basal diet was as described previously (12), except that DL-α-tocopherylacetate was added as an antioxidant to the diets (0.02% w/w). The source and the relative amount of fat are given in Table 1. Four of the diets (HLRSO, HHO, OO, TE; for explanation of abbreviations, see Table 1) were supplemented with sunflowerseed oil corresponding to 4.6 energy-% of linoleic acid. The diets were prepared by ASTRA-EWOS (Södertälje, Sweden). The animals were subjected to reversed lighting

periods (light was automatically switched on at 10 p.m. and off at 10 a.m.). Each rat received 15 g food per day and water ad lib. The rats weighed 60 g at the start of the experiment and were weighed weekly throughout the experiment. Further details of the diets are found in (1).

Fatty Acid Composition of Experimental Diets

The fatty acid composition of the experimental diets is shown in Table 2. The diets were so composed that the linoleic acid content of the sunflowerseed oil supplemented diets should be equal and well above the minimum requirement for avoiding essential fatty acid deficiency (13). No clinical signs of essential fatty acid deficiency were seen in these animals. For comparison, diets with partially hydrogenated low erucic acid rapeseed oil and partially hydrogenated herring oil but without supplement of linoleic acid were included in the experiment. These diets (HLRSO- and HHO-, respectively) had linoleic acid contents of less than 1% of total fatty acids. It was also desirable to keep the content of monounsaturated fatty acids at the same level in the different diets. However, different isomers of the monounsaturated fatty acids appear in the different diets. A detailed analysis of positional isomers of monoenoic acids in the partially hydrogenated herring oil is found in (1). The amount of *trans* isomers of monounsaturated fatty acids was varied in the diets; the highest amount was present in HLRSO- (29.7% of total fatty acids) whereas diet OO had undetectable levels.

Lipid Analysis

Total lipids were extracted from the tissues examined by the method of Folch et al. (14). Fatty acid methyl esters were prepared from

TABLE 2

Fatty Acid Composition (% w/w) of Diets Containing 20% (w/w) Fat*

	Diet					
Fatty acid[b]	HLRSO[a]	HHO[a]	OO[a]	TE[a]	HLRSO-	HHO-
14:0	0.2	5.3	0.1	1.3	0.2	7.5
14:1	–	0.6	–	0.4	–	–
15:0	–	0.5	–	0.1	–	0.6
16:0	5.0	10.8	9.6	4.0	4.7	13.6
16:1 *cis*	0.2	3.0	0.7	2.9	0.3	3.3
16:1 *trans*		3.0		0.2		3.2
17:0	0.1	0.2	0.1	0.1	0.1	–
18:0	17.9	3.0	3.4	1.5	20.9	3.1
18:1 *cis*	20.6	8.7	71.8	43.6	20.7	7.2
18:1 *trans*	35.7	4.5	0.0	2.9	43.6	6.0
18:2ω6	12.2	12.1	12.0	12.6	0.8	0.2
18:3ω6	0.1	0.1	–	–	–	–
18:3ω3	0.2	0.2	0.7	0.5	–	–
20:0	0.9	1.8	0.4	0.1	1.1	2.5
20:1 *cis*	0.3	8.0	0.3	1.6	0.3	10.6
20:1 *trans*	1.1	5.0		0.0	1.4	6.6
20:2ω6	–	0.5	–	0.2	–	–
20:3ω6	–	0.2	–	–	–	–
20:4ω6	–	0.1	–	–	–	–
22:0	0.6	2.5	0.2	0.4	0.6	3.3
22:1 *cis*	0.8	12.4	–	21.7	0.8	17.4
22:1 *trans*		7.1	–	1.5		10.1
22:2ω6	–	0.8	–	0.3	–	0.6
22:6ω3	0.2	0.1	0.1	0.1	0.2	0.1
24:1	0.2	0.7	–	0.6	0.1	1.0
Others	3.7	8.8	0.6	3.4	4.2	3.1

*Partially hydrogenated low erucic acid rapeseed oil (HLRSO + 4.6 energy-% 18:2); partially hydrogenated herring oil (HHO + 4.6 energy-% 18:2); olive oil (OO + 4.6 energy-% 18:2); trierucin + triolein (TE + 4.6 energy-% 18:2); partially hydrogenated low erucic acid rapeseed oil (HLRSO-, without 18:2 supplement); partially hydrogenated herring oil (HHO-, without 18:2 supplement).

[a]Diet was supplemented with sunflowerseed oil corresponding to 4.6 energy-% linoleic acid.

[b]The shorthand notation used for the fatty acids indicates chain length:number of double bonds. ω = First double bond position beginning from the hydrocarbon end; methylene interruption is assumed if not otherwise specified.

the total lipid extract by transesterification with methanolic sodium methoxide (0.5 M) followed by acidic esterification with methanolic hydrogen chloride (15). The fatty acid composition of total lipid extracts and phospholipid fractions was determined by glass capillary gas chromatography. The system used consisted of a Hewlett Packard 5710A gas chromatograph, equipped with an all-glass solventless injector, a FID detector, a Hewlett-Packard 3390 electronic integrator and an ABC-80 microcomputer (Luxor, Motala, Sweden) for automatic data handling. All data software were developed at this laboratory. The column was a homemade Pyrex® glass capillary column with barium carbonate deposition, coated with Silar 5CP. Further details on the gas chromatographic system are described in (16). Separation of monounsaturated fatty acid methyl esters into *cis* and *trans* isomers was achieved by reversed phase high pressure liquid chromatography. The system consisted of an Optilab 931 HSRI high performance liquid chromatograph with an interference refractive index (IRI) detector Multiref 902 (Optilab, Vällingby, Sweden). The column used was a 200 × 4.6 mm i.d. stainless steel column, slurry packed with 5μ Nucleosil C_{18} (Machery-Nagel, Düren, German Federal Republic) as described in (17).

Phospholipid Analysis

The Folch extract of washed rat platelets was further separated into a neutral lipid fraction and individual phospholipid classes by a combination of the HPLC method described by Patton et al. (18) and the TLC method published by Korte et al. (19). A quantitative determination of the different phospholipid classes was achieved by phosphorus determination (20). Phosphatidylserine (PS) and phosphatidylinositol (PI) were not completely resolved in the thin layer system used. The sum of PS and PI was therefore determined in this system. The TLC-zone containing PS+PI was extracted and PI and PS were separated by HPLC. The relative content of the two phospholipids was determined by capillary GLC using nonadecanoic acid (19:0) as an internal standard. The detection limit of our system did not permit determination of phospholipid fatty acids from individual rats. The fatty acid analysis of individual phospholipid classes was, therefore, performed on pooled material (5-6 rats) in each dietary group.

Δ^5- and Δ^6-Desaturase Assay

Δ^5- and Δ^6-Desaturase activities in liver microsomes were determined as described in (8). In essence the procedure was the following: Livers were removed immediately after sacrifice of the animals and rinsed in ice-cold phosphate buffer pH 7.0. After homogenization in four volumes of buffer, the homogenate was centrifuged at 20,000 × g for 10 min. The supernatant was further centrifuged at 100,000 × g for 60 min to obtain microsomes. The pellet was resuspended in buffer to a protein concentration of 3 mg microsomal protein/ml. Protein determinations were performed according to Lowry et al. (21). All steps of the preparation were carried out at 4 C. Microsomes corresponding to 1 mg of protein were incubated with 100 nmoles of fatty acid (containing 0.5 μCi of ^{14}C label) at 37 C for 20 min. Termination of the reaction was achieved by addition of 1 ml of 5% HCl in methanol. The substrate and product fatty acids were separated, after esterification, by reversed phase high pressure liquid chromatography.

Platelet Aggregation

Platelet aggregation was studied in a Payton aggregometer type 300 BD-5 (Payton Associates Limited, Scarborough, Ontario, Canada). The temperature was set at 37 C and the stirring system at 900 rpm.

Preparation of Washed Rat Platelets

Rats were anesthetized with diethyl ether. Nine ml blood was drawn from the aorta into a plastic syringe containing 1 ml 0.077 M EDTA-Na_2. Platelet rich plasma (PRP) was prepared by centrifugation at 200 × g for 15 min at room temp. The PRP was carefully aspirated and recentrifuged at 1000 × g for 20 min. The supernatant was discarded and the platelet pellet washed once with 0.154 M NaCl-25 mM Tris-0.2 mM EDTA-Na_2, pH 7.4. The platelet suspension was centrifuged again at 1000 × g for 20 min, and the pellet was finally resuspended in 0.134 M NaCl-15 mM Tris-5mM D-glucose, pH 7.4. The platelets were counted in a Bürker chamber and the platelet count was adjusted to 1×10^9 platelets/ml with the glucose containing buffer.

Thrombin Stimulation of Washed Rat Platelets

Stimulation of washed rat platelets with thrombin was carried out in an aggregometer cuvette at 37 C. The platelet suspension (0.5 ml) was first preincubated for 2 min at 37 C in the stirred cuvette. Thrombin (2.5 U, dissolved in 8.3 μl 0.9% NaCl solution) was added and the incubation was continued for 10 min at 37 C. Termination of the reaction was accomplished by addition of 3 ml of ethanol. For the quantitative determination of 12-hydroxy-5,8,10, 14-eicosatetraenoic acid (12-HETE) and 12-

hydroxy-5,8,10-heptadecatrienoic acid (HHT), 0.95 μg of 13-hydroxy-6,9,11-octadecatrienoic acid was added as an internal standard.

Determination of 12-HETE and HHT

The reaction mixtures from the thrombin stimulations were acidified and extracted twice with diethyl ether. The ether extract was washed with water and evaporated to dryness. The residue was dissolved in 100 μl hexane/2-propanol/acetic acid (98.5:1.5:0.04, v/v/v). Analysis of 12-HETE and HHT in the extract was done in a straight phase HPLC system with hexane/2-propanol/acetic acid (98.5:1.5:0.04, v/v/v) as mobile phase. The UV absorbance was monitored at 234 nm (Hamberg, M., unpublished).

Determination of 6-Keto-Prostaglandin $F_{1\alpha}$

The aorta was dissected free from surrounding tissue and cut into small pieces. Aorta pieces were incubated in 50 mM Tris buffer, pH 7.4 for 30 min at 37 C. The incubation was terminated by freezing the samples in CO_2/ethanol, and 6-keto-prostaglandin $F_{1\alpha}$ was determined in the incubate by a commercial RIA-kit (Seragen, Boston, Massachusetts).

RESULTS

Body Weight, Growth, Food Consumption and Food Efficiency of Rats

The effect of the different experimental diets on body weight, growth, food consumption and food efficiency is summarized in Table 3. The average food consumption was 15.9 g per day per rat, and there were small differences among the dietary groups. Rats fed the diets without linoleic acid supplement (HLRSO- and HHO-) had significantly lower body weights after 10 weeks on the diet than did the linoleic acid supplemented group OO.

Hematological Data

There were small variations in platelet count except for group TE, which had a reduction of 10% compared to group OO ($670 \pm 24 \times 10^9$ platelets/l, versus $746 \pm 51 \times 10^9$ platelets/l, TE: n = 5, OO: n = 6). There were no significant differences in hemoglobin concentration or in the number of erythrocytes or leukocytes among the groups.

Effect of Dietary Oils on Fatty Acid Composition of Liver Microsomes

In previous work we have determined the fatty acid composition of the phospholipid fraction of liver microsomes in rats receiving different dietary oils, one of them being partially hydrogenated herring oil (HHO) (8). In the course of the present study we found that the phospholipid fatty acid distribution of liver microsomes did not differ significantly from the fatty acid distribution in the total lipid extract of the microsomes. In the present study, therefore, we have limited our determination of microsomal fatty acids to the total lipid extract.

The fatty acid composition of total lipid extracts of liver microsomes for five of the experimental groups is shown in Table 4. Considerable amounts of *trans* isomers of octadecenoic acids were incorporated into the liver microsomes. The degree of incorporation was not dependent on the linoleic acid content of the diet, as can be seen in Table 4 for dietary groups HLRSO and HLRSO-, receiving 12.2% and 0.8% of linoleic acid, respectively (Table 2). The linoleic acid content was considerably higher in groups HLRSO and HHO than in group OO. The elongation and desaturation product of linoleic acid, arachidonic acid (20:4ω6), was not significantly different in the HLRSO and OO groups, however. Group HRLSO- showed a profound decrease in the arachidonic acid content. The

TABLE 3

Body Weight, Growth, Food Consumption and Food Efficiency of Rats

Group	Body weight		Growth (g/day)	Food consumption (g/rat day)	Food efficiency %
	Start	10 weeks			
HRLSO	61 ± 2[a]	378 ± 14	4.46	16.3	29
HHO	62 ± 2	415 ± 29	5.19	16.8	31
OO	61 ± 3	389 ± 13	4.82	14.9	32
TE	60 ± 2	356 ± 38	4.35	14.7	30
HLRSO-	59 ± 3	355 ± 16[b]	4.32	16.8	26
HHO-	60 ± 2	351 ± 11[c]	4.16	16.1	26

[a]Mean ± standard deviation (n = 6-20).

[b,c]Values are significantly different from group OO with $p<0.005$ and $p< 0.001$, respectively.

TABLE 4

Fatty Acid Composition of Total Lipids of Rat Liver Microsomes of Rats Fed Different Diets for 10 Weeks

Fatty acid	Diet				
	HLSRO	HHO	OO	TE	HLRSO-
16:0	7.1 ± 0.4[a]	11.8 ± 0.2	12.5 ± 0.6	10.5 ± 0.9	8.7 ± 0.3
16:1	1.7 ± 0.1	2.7 ± 0.8	0.6 ± 0.1	1.3 ± 0.2	2.0 ± 0.3
17:0	0.1 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	trace[b]
18:0	20.9 ± 1.1	18.1 ± 0.9	23.2 ± 0.5	15.6 ± 0.7	21.5 ± 0.6
18:1 *cis*	8.1 ± 0.5	7.6 ± 0.6	15.0 ± 1.0	16.7 ± 1.1	17.5 ± 0.7
18:1 *trans*	12.6 ± 0.9	8.3 ± 0.7	0.6 ± 0.1	6.7 ± 0.4	11.7 ± 0.5
18:2ω9	— —	— —	— —	— —	3.3 ± 0.2
18:2ω6	11.3 ± 1.1	14.7 ± 1.3	6.9 ± 0.4	7.5 ± 0.6	5.7 ± 0.3
18:3ω6	0.2 ± 0.1	0.2 ± 0.1	trace	0.1 ± 0.1	trace
20:0	trace	0.2 ± 0.1	trace	trace	trace
20:1	0.2 ± 0.1	1.7 ± 0.1	0.8 ± 0.1	1.6 ± 0.2	0.3 ± 0.1
20:2ω9	— —	trace	— —	trace	0.4 ± 0.1
20:2ω6	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	— —
20:3ω9	0.2 ± 0.1	0.3 ± 0.1	0.7 ± 0.1	0.9 ± 0.3	6.3 ± 0.6
20:3ω6	0.7 ± 0.1	2.0 ± 0.2	0.7 ± 0.1	1.1 ± 0.3	1.0 ± 0.1
20:4ω6	29.9 ± 0.4	23.6 ± 1.4	30.6 ± 0.6	28.9 ± 1.4	7.0 ± 0.5
20:5ω3	— —	trace	trace	trace	0.2 ± 0.1
22:0	— —	trace	trace	trace	0.3 ± 0.1
22:1	trace	0.3 ± 0.1	trace	0.5 ± 0.1	trace
22:4ω6	0.6 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.1 ± 0.1
22:5ω6	1.3 ± 0.3	0.8 ± 0.3	0.7 ± 0.1	0.8 ± 0.2	1.4 ± 0.2
22:5ω3	0.3 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	trace
22:6ω3[c]	2.9 ± 0.2	3.0 ± 0.2	4.7 ± 0.4	2.9 ± 0.1	5.0 ± 0.2
Others	1.7 ± 0.1	3.2 ± 0.5	1.5 ± 0.2	3.1 ± 0.2	6.7 ± 0.2

[a]Mean ± standard deviation (n=5).

[b]Trace = < 0.1%.

[c]Coincides with 24:0.

group fed hydrogenated herring oil (HHO) accumulated linoleic acid compared to the rest of the dietary groups and displayed a significant reduction in arachidonic acid content compared to the other sunflowerseed oil supplemented groups.

In the unsupplemented group HLRSO-, 20:3ω9 constituted more than 6% of total fatty acids. Calculation of the 20:3ω9 to 20:4ω6 ratio gives a value of 0.79, indicative of essential fatty acid deficiency (13). The sum of polyunsaturated ω6 fatty acids in group HLRSO- was only 16.18% of total fatty acids, as compared to around 40% in the linoleic acid supplemented groups.

Incorporation of 20:1 was observed in group HHO and in group TE. The partially hydrogenated herring oil in diet HHO contained large amounts of isomeric eicosenoic acids (1) which could account for this incorporation. Diet TE, however, contained only 1.6% of 20:1 compared to 13.0% for diet HHO (Table 2), which suggests that other mechanisms are involved in this case. Only small amounts of docosenoic acids were incorporated into the liver microsomes of group HHO and TE in spite of the great abundance of these acids in the corresponding diets.

Effect of Dietary Oils on Microsomal Δ^6- and Δ^5-Desaturase Activity

The in vitro Δ^6- and Δ^5-desaturase assays were performed at saturated substrate levels (8). The results from the determination of Δ^6- and Δ^5-desaturase activities in liver microsomes from rats fed different experimental diets are shown in Figures 1 and 2, respectively.

There were no significant differences in the Δ^6-desaturase activity of liver microsomes from rats fed linoleic acid supplemented diets. The Δ^6-desaturase activity rose about 3-fold in the microsomes from the two unsupplemented groups, HLRSO- and HHO-.

The Δ^5-desaturase activity was higher than the Δ^6-desaturase activity in all groups. The partially hydrogenated rapeseed oil group (HLRSO) had significantly higher Δ^5-desaturase activity than the olive oil group (OO). As was seen with the Δ^6-desaturase activity, the Δ^5-desaturase activity rose drastically in the microsomes from the two unsupplemented groups. Interestingly, the interrelation between groups HLRSO and HHO in Δ^5-desaturase activity still remained the same in the corresponding unsupplemented groups (HLRSO- and HHO-).

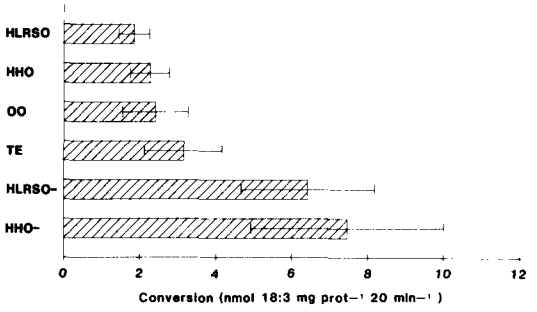

FIG. 1. Influence of dietary oils on Δ^6-desaturase activity in rat liver microsomes. Linoleic acid (18:2ω6) was used as substrate for the reaction. Bars represent the mean (n=5) and horizontal lines represent ±S.D.

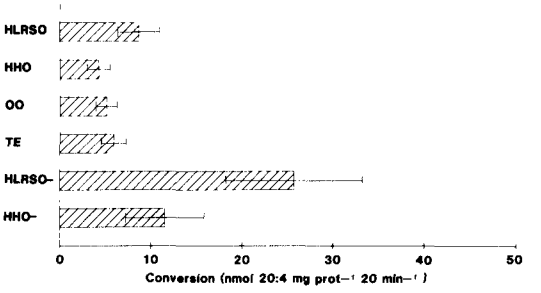

FIG. 2. Influence of dietary oils on Δ^5-desaturase activity in rat liver microsomes. Dihomo-γ-linolenic acid (20:3ω6) was used as substrate for the reaction. Bars represent the mean (n=5) and horizontal lines represent ±S.D.

Effect of Dietary Oils on the Fatty Acid Composition of Platelet Total Lipids

The results from the determination of fatty acid composition of platelet total lipids in rats fed different diets for 10 weeks are given in Table 5. Substantial incorporation of *trans* fatty acids was observed in platelets from rats fed *trans* fatty acid containing diets. The highest levels were found in the groups with and without sunflowerseed oil supplemented partially hydrogenated rapeseed oil (HLRSO and HRLSO-). There was an increase in linoleic acid content in the groups HRLSO and HHO compared to group OO, whereas a decreased content was found in group HLRSO-. Eicosenoic and docosenoic acids were incorporated into platelet

TABLE 5

Fatty Acid Composition of Total Lipids of Platelets Isolated from Rats Fed Different Diets for 10 Weeks

	Diet				
Fatty acid	HLRSO	HHO	OO	TE	HLRSO-
12:0	0.4 ± 0.3[a]	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.2
14:0	0.7 ± 0.2	1.2 ± 0.1	0.6 ± 0.2	0.9 ± 0.1	0.8 ± 0.1
14:1	0.1 ± 0.1	trace[b]	trace	trace	0.2 ± 0.1
16:0	17.9 ± 0.7	23.0 ± 2.6	25.1 ± 0.4	23.4 ± 0.2	17.1 ± 1.0
16:1	0.8 ± 0.2	1.6 ± 0.5	0.2 ± 0.1	0.5 ± 0.1	1.0 ± 0.1
18:0	14.9 ± 0.6	11.6 ± 0.2	14.4 ± 0.2	11.7 ± 0.2	12.7 ± 0.4
18:1 *cis*	9.8 ± 0.4	7.5 ± 0.1	13.1 ± 0.9	13.2 ± 1.0	14.2 ± 1.1
18:1 *trans*	5.5 ± 0.5	1.8 ± 0.2	– –	1.9 ± 0.1	7.0 ± 1.0
18:2ω6	6.2 ± 0.5	6.8 ± 0.3	4.5 ± 0.4	5.1 ± 0.5	3.5 ± 0.6
18:3ω6	trace	trace	trace	trace	trace
18:3ω3	trace	trace	trace	0.1 ± 0.1	trace
20:0	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.1 ± 0.1	0.4 ± 0.1
20:1	1.1 ± 0.1	2.7 ± 0.2	1.3 ± 0.1	1.7 ± 0.1	1.7 ± 0.2
20:2ω6	0.6 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	– –
20:3ω9	0.1 ± 0.1	0.1 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	6.6 ± 0.3[c]
20:3ω6	0.5 ± 0.1	0.1 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.9 ± 0.1
20:4ω6	22.5 ± 1.3	20.9 ± 2.3	22.4 ± 1.2	19.8 ± 1.5	10.6 ± 0.6
20:5ω3	trace	trace	trace	trace	0.4 ± 0.1
22:0	0.4 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
22:1	0.7 ± 0.1	3.8 ± 0.2	0.5 ± 0.1	4.8 ± 0.4	0.5 ± 0.2
22:3ω9	0.1 ± 0.1	0.1 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	1.2 ± 0.1
22:4ω6	4.6 ± 0.4	3.4 ± 0.4	3.5 ± 0.6	3.2 ± 0.5	0.4 ± 0.1
22:5ω6	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.1
22:5ω3	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	trace	0.1 ± 0.1
22:6ω3	0.4 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.7 ± 0.1
Others	11.9 ± 1.7	11.8 ± 0.9	10.8 ± 1.2	10.9 ± 0.6	19.0 ± 1.0

[a]Mean ± standard deviation (n = 5).

[b]Trace = <0.1%.

[c]Coincides with 20:2ω6.

lipids of all dietary groups. Eicosenoic acid was especially abundant in group HHO, probably reflecting the high 20:1 content of the corresponding diet. However, group OO also contained eicosenoic acid although very low levels were present in the diet. Docosenoic acids were incorporated into the platelet lipids mainly in groups HHO and TE. The corresponding diets are rich in these docosenoic acids (Table 2). The arachidonic acid content varied little in-the platelets of sunflowerseed oil supplemented groups. A somewhat lower content, however, was found in group TE. Platelets from group HLRSO- (unsupplemented) contained only half as much arachidonic acid as platelets from sunflowerseed oil supplemented groups. Platelets from group HLRSO- also had a much reduced content of the elongation product of arachidonic acid, 22:4ω6. The capillary column used for this analysis was not able to separate 20:2ω6 and 20:3ω9. There was, however, a significantly increased content of 20:2ω6 + 20:3ω9 in the platelet lipids of group HRLSO- compared to the sunflowerseed oil supplemented groups, indicating the presence of 20:3ω9. The content of another ω9 fatty acid, 22:3ω9, was also higher in group HLRSO- compared to all other groups. This further strengthens the assumption of increased ω9 fatty acid content in this group.

TABLE 6

Fatty Acid Composition (% w/w) of Platelet Phosphatidylethanolamine of Rats Fed Different Dietary Oils for 10 Weeks

	Diet				
Fatty acid	HLRSO	HHO	OO	TE	HLRSO-
16:0	3.3	4.3	5.7	8.0	2.3
16:1	0.9	0.9	0.2	0.6	0.8
18:0	11.3	9.7	11.4	10.5	10.9
18:1 *cis*	8.5	7.0	9.9	11.7	8.3
18:1 *trans*	1.6	0.6	–	0.7	5.8
18:2ω9	–	–	–	–	1.0
18:2ω6	2.2	3.2	1.8	2.2	2.7
18:3ω6	–	–	–	–	–
18:3ω3	–	0.1	–	0.1	–
20:0	0.2	0.2	0.2	–	0.2
20:1	0.7	3.0	1.2	1.5	1.8
20:2ω9	0.1	–	–	–	0.9
20:2ω6	0.5	0.8	0.6	0.8	–
20:3ω9	–	0.1	0.3	0.3	12.3
20:3ω6	0.4	0.9	0.4	0.5	1.3
20:4ω6	35.4	36.6	36.4	31.9	20.1
20:5ω3	–	0.2	0.1	0.1	0.9
22:0	0.2	0.3	0.2	0.1	–
22:1	–	2.4	0.3	3.2	0.2
22:3ω9	–	–	0.4	0.3	2.5
22:4ω6	11.5	9.5	7.9	8.3	1.1
22:5ω6	0.5	0.3	0.3	0.2	0.7
22:5ω3	0.2	0.4	0.3	0.1	0.3
22:6ω3	0.4	0.4	0.5	0.4	1.0
24:1	–	0.1	0.1	0.3	–
Others	22.1	19.0	21.8	18.2	24.9

Phospholipid Distribution in Rat Platelets

By the combination of TLC and HPLC the total lipid extract of pooled rat platelets was separated into phospholipid classes. There were only minor differences in the phospholipid distribution between the different dietary groups (data not shown). Phosphatidylinositol was the smallest component, comprising about 5% of total phospholipids.

Effect of Dietary Oils on the Fatty Acid Composition of Platelet Phospholipids

The fatty acid compositions of platelet phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine of rats fed different dietary oils for 10 weeks are shown in Tables 6-8.

The unsupplemented hydrogenated rapeseed oil group (HLRSO-) deviated in its fatty acid composition compared to the other dietary groups in all phospholipid fractions analyzed. Although the linoleic acid content in the different fractions generally was not lower than for the other diets, higher ω6 metabolites, e.g. arachidonic acid and 22:4ω6, were significantly reduced in all phospholipid fractions. Concomitantly, considerable amounts of ω9 fatty acids accumulated, especially 20:3ω9 but also 18:2ω9 and 22:3ω9. In phosphatidylinositol, for example, 20:3ω9 constituted 17.3% of total fatty acids, almost as much as the arachidonic acid content (18.6%). The phospholipid fractions from group HLRSO- contained higher levels of ω3 fatty acids than all other dietary groups. This pattern with lower levels of ω6 fatty acids and higher levels of ω3 and ω9 fatty acids was evident in all phospholipid fractions from group HLRSO-. Eicosenoic acid was incorporated into the phospholipid fractions of all dietary groups. The incorporation, however, was not proportional to the dietary content. Docosenoic acid appeared in appreciable amount in platelet phospholipids, in contrast to the liver microsomes. The highest relative incorporation was found in phosphatidylserine, which incorporated significant amounts of other long chain monounsaturated fatty acids as well. Strikingly high was the incorporation of 22:1 in the phosphatidylserine fractions from the dietary groups HHO and TE. Phosphatidylserine from group HHO contained 6.7% 22:1 and the corresponding figure for group TE was 13.5%. Interestingly, phosphatidylinositol incorporated very small amounts of docosenoic acids, in contrast to all other fractions analyzed.

TABLE 7

Fatty Acid Composition (% w/w) of Platelet Phosphatidylinositol of Rats Fed Different Dietary Oils for 10 Weeks

	Diet				
Fatty acid	HLRSO	HHO	OO	TE	HLRSO-
16:0	1.9	4.0	3.9	3.4	3.6
16:1	0.6	0.1	0.4	0.7	0.7
18:0	34.1	33.1	37.2	34.1	29.6
18:1 *cis*	9.6	7.2	7.1	8.6	8.6
18:1 *trans*			–	1.0	5.7
18:2ω9	–	–	–	–	1.5
18:2ω6	0.9	1.8	1.1	1.3	0.7
18:3ω6	0.1	–	–	–	0.1
18:3ω3	–	–	–	–	–
20:0	0.2	0.3	0.2	0.1	0.1
20:1	0.3	1.6	0.6	1.0	0.8
20:2ω9	–	–	–	–	0.2
20:2ω6	0.2	0.4	0.5	0.3	–
20:3ω9	0.4	0.4	1.0	1.1	17.3
20:3ω6	0.6	1.5	0.6	0.8	1.0
20:4ω6	40.8	42.8	40.4	39.4	18.6
20:5ω3	–	0.1	0.1	0.1	0.4
22:0	0.1	0.1	–	–	0.3
22:1	–	0.5	0.9	0.7	–
22:3ω9	–	0.1	0.1	–	1.0
22:4ω6	3.6	3.2	2.1	2.6	0.7
22:5ω6	0.3	0.2	0.2	0.2	0.4
22:5ω3	0.4	0.3	0.1	0.9	0.9
22:6ω3	0.3	0.3	0.9	0.7	0.9
24:1	0.2	–	0.1	–	0.1
Others	5.4	2.0	2.5	3.0	6.8

TABLE 8

Fatty Acid Composition (% w/w) of Platelet Phosphatidylserine of Rats Fed Different Dietary Oils for 10 Weeks

	Diet				
Fatty acid	HLRSO	HHO	OO	TE	HLRSO-
16:0	1.9	2.3	2.3	3.2	5.5
16:1	0.7	0.7	0.5	0.7	1.0
18:0	24.0	24.5	30.5	22.0	22.6
18:1 *cis*	8.5	10.8	14.8	17.7	17.0
18:1 *trans*	7.5	3.2	–	–	7.6
18:2ω9	–	–	–	–	1.5
18:2ω6	9.3	11.6	5.3	5.3	6.0
18:3ω6	–	–	–	–	–
18:3ω3	–	0.1	0.1	0.1	–
20:0	1.6	0.7	1.6	–	1.5
20:1	2.7	3.8	2.9	1.9	3.5
20:2ω9	–	–	–	–	0.3
20:2ω6	1.5	1.5	1.1	1.1	–
20:3ω9	–	0.1	0.2	0.2	4.3
20:3ω6	0.5	1.4	0.7	0.7	1.2
20:4ω6	29.1	27.7	28.9	25.7	14.2
20:5ω3	–	–	–	–	0.2
22:0	1.5	0.8	1.6	0.5	1.0
22:1	3.3	6.7	2.3	13.5	2.4
22:3ω9	–	–	0.7	0.3	1.8
22:4ω6	3.6	1.8	3.0	2.6	0.4
22:5ω6	0.3	–	0.2	0.2	0.3
22:5ω3	0.5	0.2	0.2	0.1	0.6
22:6ω3	1.0	0.3	0.9	1.1	2.4
24:1	1.1	0.3	0.9	1.3	0.7
Others	1.4	1.5	1.3	2.1	4.0

Effects of Dietary Oils on the Production of 12-Hydroxy-5,8,10,14-Eicosatetraenoic Acid, 12-Hydroxy-5,8,10-Heptadecatrienoic Acid and 6-Ketoprostaglandin $F_{1\alpha}$

Washed rat platelets were stimulated with thrombin in order to liberate arachidonic acid for biosynthesis of 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) and 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT). A high dose of thrombin (5 U/ml) was used in this experiment to set free arachidonic acid maximally to assess the effects of dietary oils on the arachidonic acid metabolizing enzymes in rat platelets. No difference in the aggregation response of the platelets from the different dietary groups was observed at this high thrombin concentration. The results of the determination of 12-HETE and HHT in thrombin stimulated platelets from rats receiving different dietary oils are shown in Figures 3 and 4, respectively. There were small differences in the 12-HETE production in platelets from groups HLRSO, HHO and OO. Group TE showed a slightly decreased synthesis of 12-HETE compared to group OO ($p<0.05$). The two groups not supplemented with sunflowerseed oil produced only half as much 12-HETE as group OO ($p<0.005$).

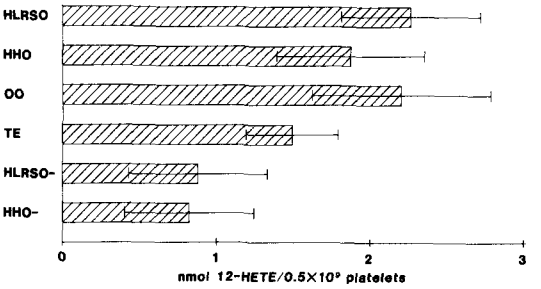

FIG. 3. Influence of dietary oils on 12-HETE production in stimulated rat platelets. Bars represent the mean (n=5) and horizontal lines represent ±S.D.

The same pattern was seen for the synthesis of HHT. In this case, group TE did not show a statistically significant decrease compared to group OO. The unsupplemented groups HRLSO- and HHO- produced only 50% HHT compared to the production of group OO ($p<0.05$).

The effect of dietary oils on the production of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-PGF-$_{1\alpha}$) from endogenous arachidonic acid was studied in aorta slices. The result from the radioimmunologic analysis of 6-keto-PGF$_{1\alpha}$ is shown in Fig-

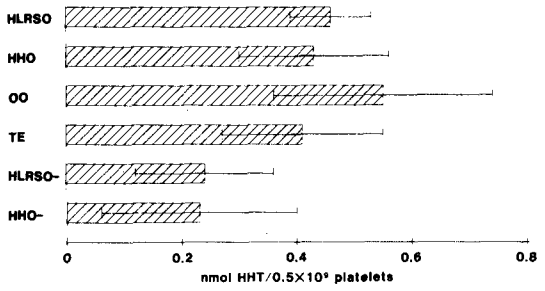

FIG. 4. Influence of dietary oils on HHT production in stimulated rat platelets. Bars represent the mean (n=5) and horizontal lines represent ±S.D.

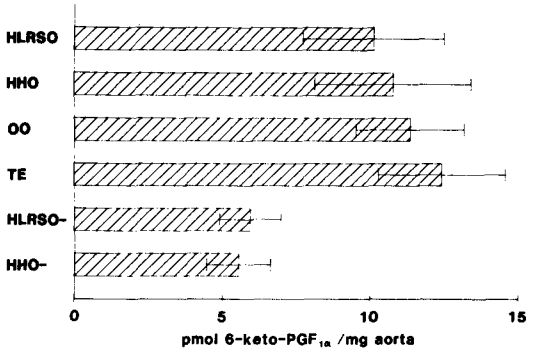

FIG. 5. Influence of dietary oils in 6-keto-prostaglandin $F_{1\alpha}$ production of rat aorta incubates. Bars represent the mean (n=5) and horizontal lines represent ±S.D.

ure 5. No statistically significant differences were observed between the sunflowerseed oil supplemented groups. The two unsupplemented groups produced approximately half the amount of 6-keto-$PGF_{1\alpha}$ compared to group OO ($p<0.00005$ and $p<0.005$ for HLRSO- and HHO-, respectively).

DISCUSSION

It has been reported that dietary mixed isomeric *cis* and *trans* octadecenoates suppress arachidonic acid in rat tissues (22). In the present study, two of the sunflowerseed oil supplemented diets contained high amounts of *trans* fatty acids, mainly octadecenoic acids. These diets, HLRSO and HHO, contained 20.2% and 12.2% *trans* monoenes, respectively (Table 2). Analysis of liver microsomes from rats fed diet HLRSO did not differ in arachidonic acid content compared to the olive oil group (OO). The group receiving HHO, however, showed a significant reduction in arachidonic acid content compared to group OO ($p<0.0001$). Platelets from the two groups HRLSO and HHO did not differ in the arachidonic acid content (Table 5). The reduced amount of arachidonic acid in liver microsomes from group HHO could be due to inhibition of arachidonic acid synthesis or an increased arachidonic acid metabolism, for example by an increased peroxisomal oxidation. An inhibition of the biosynthesis seems probable in this case, since both linoleic acid and eicosa-8,11,14-trienoic acid were significantly accumulated in the liver microsomes of group HHO compared to group OO ($p<0.0001$ and $p<0.00005$, respectively). It is known that *cis* octadecenoic acids inhibit Δ^6- as well as Δ^5-desaturase activity in rat liver microsomes (7). Although the *trans* octadecenoic acid content of diet HHO was considerably lower than that of diet HRLSO, the total amount of monoenoic acids was approximately the same; differences in the isomeric or chain-length distribution could explain the different effects of the two diets on the arachidonic acid level in liver microsomes.

In a recent paper, Gómez Dumm et al. reported that rats maintained on a fat-free diet exhibited a significant reduction in Δ^5-desaturase activity compared to animals on a balanced diet (23). In the present study two dietary groups were included that did not get linoleic acid supplementation. Tissues from these groups, HLRSO- and HHO-, had a marked reduction in arachidonic acid content and had accumulated 20:3ω9. Group HLRSO- had a 20:3ω9 to 20:4ω6 ratio of 0.79 in the total lipids of liver microsomes, indicating an essential fatty acid deficiency (13). The Δ^5-desaturase activity in these liver microsomes was five-fold higher than the Δ^5-desaturase activity in microsomes from group OO (Fig. 2) and three-fold higher than the activity in the corresponding linoleic acid supplemented group (HRLSO). A similar increase in Δ^5-desaturase activity was seen in microsomes from group HHO- compared to group HHO. In our study the animals were not raised on a fat-free diet but the two groups without linoleic acid supplementation showed retarded growth and had a high triene to tetraene ratio, both indications of essential fatty acid deficiency. Furthermore, our data are in agreement with those of Privett et al. (24), who found depressed Δ^5- and Δ^6-desaturase activity in liver microsomes from linoleic acid supplemented animals compared to animals on a fat-free diet. The Δ^5-desaturase activity was dependent on a factor additional to that of linoleic acid content, as can be seen in Figure 2. The increase in the unsupplemented group HHO- was not as great as for group HLRSO- in absolute terms, but the relation between HLRSO

and HHO was the same as for the corresponding unsupplemented groups HLRSO- and HHO-. The Δ^5-desaturase activity in group HHO was significantly lower than that of group HLRSO. This big difference was not seen in the Δ^6-desaturase activity. A pronounced enhancement of the Δ^6-desaturase activity was observed for the linoleic acid deficient groups. Both Δ^6- and Δ^9-desaturase activities in cultured human skin fibroblasts were reported to be enhanced by growing the cells in a lipid-free medium (25). There was no clearcut relation between the Δ^6-desaturase activity and the arachidonic acid level in the liver microsomes or in the platelets (cf. Fig. 1 and Table 4 and 5). Diet HHO contained less *trans* fatty acids than diet HLRSO but, in addition, it contained considerable amounts of 20:1 and 22:1 isomers which were not present in diet HLRSO. There was a small but significant accumulation of 20:1 and 22:1 isomers in the liver microsomes of group HHO compared to group HRLSO and group OO (Table 4). These long-chain monoenes might contribute to the differences seen in Δ^5-desaturase activity between groups HLRSO and HHO (8,26). Eicosenoic acid accumulated in the liver microsomes of group TE to approximately the same level as in group HHO. Diet TE, however, contained very little eicosenoic acid compared to diet HHO. Therefore, the eicosenoic acid in group TE probably originated from dietary docosenoic acid which had been chain-shortened.

Platelets rapidly take up and release fatty acids from the surrounding medium (27). Both de novo synthesis and chain elongation of fatty acids are operative in platelets (28). It seems, though, that platelets have a limited ability to desaturate and elongate linoleic acid to arachidonic acid (29). Therefore, platelets depend on the availability of polyunsaturated fatty acids coming either from the diet or from other organs with active desaturase systems, for example the liver. Both ω3 and ω6 polyunsaturated fatty acids are incorporated into platelet phospholipids (30) and dietary manipulation could, therefore, affect the platelet fatty acid composition significantly (31).

In the present study dietary long-chain monounsaturated fatty acids accumulated in the platelet lipids. Eicosenoic acids were incorporated especially in lipids from group HHO, reflecting the high content of these isomers in the diet. Docosenoic acids accumulated in groups HHO and TE. It is interesting to note that docosenoic acid seems to be preferentially incorporated into phosphatidylserine. It has been reported earlier that erucic acid (22:1ω9) showed a special affinity for cardiolipin in heart lipids (2). In platelet phosphatidylinositol only a small incorporation of docosenoic acids was seen.

It has been reported that peroxisomes, purified from rat liver, are capable of oxidizing long-chain acyl-CoA esters (32). In a previous investigation (1) we found that rats fed partially hydrogenated marine oils accumulated less docosenoic acid in the myocardium than could be expected from the dietary content of this fatty acid. This finding suggested that the peroxisomal chain-shortening system might be of importance in the metabolism of dietary long-chain isomeric fatty acids that are not well oxidized by mitochondria (33). In spite of this auxiliary mechanism for oxidation, the data indicate that a variety of *cis* and *trans* isomers of long-chain fatty acids was incorporated into the mitochondrial membrane phospholipids after feeding partially hydrogenated marine oils. In the present study we also found a variety of long-chain isomeric fatty acids incorporated into platelet membrane phospholipids after feeding partially hydrogenated vegetable and marine oils, indicating that the peroxisomal beta-oxidizing system has a limited capacity to oxidize certain fatty acids when the influx of these acids is too high.

The production of 12-HETE was significantly lower in group TE than in group OO ($p<0.05$). Erucic acid has been reported to inhibit both soybean and peanut lipoxygenases (34). Although the arachidonic acid content in the total lipids of platelets from group TE was lower than in group OO ($p<0.05$), there were no significant differences in arachidonic acid content in phosphatidylinositol from the two groups. This does not support a phospholipase C mechanism of arachidonic acid liberation because phosphatidylinositol seems to be degraded nearly exclusively by phospholipase C (10). The production of 12-HETE by washed platelets upon thrombin stimulation was proportional to the arachidonic acid content in the platelet total lipids for all the dietary groups examined. In contrast to the liver microsomes, the arachidonic acid level was not significantly different in platelets from group HHO compared to group OO. The linoleic acid deficient group HLRSO- had, however, very low levels of arachidonic acid in the platelet lipids. This decrease was evident in all phospholipid classes analyzed. The production of both cyclooxygenase and lipoxygenase products in platelets was significantly decreased in the linoleic acid deficient groups. There was a tendency for decreased HHT formation in platelets from group TE but the difference was not statistically significant. The production of 12-HETE, HHT and 6-keto-prostaglandin $F_{1\alpha}$ did not differ between

groups HLRSO and OO. Group HLRSO accumulated significant amounts of *trans* octadecenoates, but these obviously did not interfere with prostaglandin synthesis. This finding has been reported earlier (35). The production of 6-keto-prostaglandin $F_{1\alpha}$ in rat aorta was not decreased in group TE compared to group OO, but the linoleic acid deficient groups produced significantly less than the supplemented groups. This is in accordance with earlier work which showed that EFA deficiency results in reduction of PGI_2 production by heart and aorta and thromboxane production in blood platelets (36). In the linoleic acid deficient group HLRSO-considerable amounts of 20:3ω9 accumulated in platelet phospholipids. In phosphatidylinositol, for example, this fatty acid constituted 17% of total fatty acids, almost as much as the arachidonic acid content. 20:3ω9 has been reported to inhibit cyclooxygenase (37).

In conclusion, the results of the present feeding experiment demonstrate that dietary fatty acids are expressed in the fatty acid composition of lipids from liver microsomes and platelets. Significant amounts of *trans* isomers of octadecenoic acid were incorporated both in liver and platelet lipids. Docosenoic acid was preferentially incorporated into platelet phosphatidylserine. In platelet phosphatidylinositol only very little docosenoic acid was recovered. Feeding rats with partially hydrogenated rapeseed oil resulted in an increased hepatic Δ^5-desaturase activity compared to rats fed olive oil. There was, however, no difference in the arachidonic acid content in liver microsomes from the two groups. The Δ^6-desaturase activity was not influenced significantly by the different linoleic acid supplemented diets. Both Δ^5- and Δ^6-desaturase activities were greatly enhanced by linoleic acid deficient diets. It thus seems that high levels of dietary monoenoic *trans* fatty acids do not interfere seriously with arachidonic acid synthesis as long as a sufficient amount of linoleic acid is available. Nor did the isomeric long-chain fatty acids influence further metabolism of arachidonic acid into 12-HETE and HHT in platelets when linoleic acid was supplemented simultaneously. One should, however, be very careful in extrapolating these results to other enzyme-systems because the membrane fatty acid composition was strongly influenced by the dietary regimes.

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Temperature-Induced Phase Change in a Fat. A Study by Electron Spin Resonance

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ABSTRACT

Electron Spin Resonance, Differential Scanning Calorimetry and rheological techniques have been used to study the physical changes induced by temperature in lard and in the solid and liquid fractions obtained by fractionation of lard at 15 C. The mobilization process of a C18 fatty acid nitroxide derivative dispersed in the molten fat has been observed in the temperature range −50 to +70 C.

The mobilization of the probe seemed to be concomitant with the melting of the low melting point glycerides. Above this temperature, all the probes were in the liquid phase and their mobility reflected the viscosity of their liquid environment, or the viscosity of the bulk fat when crystal was no longer present.

Probe mobility was temperature dependent, and it was identical for the three fats at the same temperature, despite their different triglyceride compositions.

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INTRODUCTION

During heating of a complex fat like lard, polymorphic transitions and different melting phenomena follow one another. Thermal analysis is the method most commonly used to study these problems, though sometimes this technique does not allow distinction of an endothermic polymorphic transition from a fusion. Moreover, because of the succession of melting of the different triglyceridic groups, it is sometimes difficult to distinguish the limits of these melting ranges. Nuclear magnetic resonance (NMR) is sensitive to changes in the solid fat content, but sophisticated methods are necessary to study polymorphic transitions (1).

Raison et al. (2) and Mehlhorn et al. (3) have studied the phase changes occurring in lipid systems by spin probing. This method has been used essentially for oriented systems like biological membranes, but few investigations have been carried out on pure lipids. However, Mehlhorn et al. (3) have shown that nitroxide fatty acid dissolved in highly purified lipid systems behaved like impurities due to the fact that a crystal cannot form in the neighborhood of the probe. This molecule may be embedded in local regions of supercooled liquid.

Electron spin resonance (ESR) spectra are sensitive to the physical state and to the viscosity of these microdomains.

Probe mobility is generally described by one of these two parameters:

- S = order parameter used when nitroxides are moving anisotropically. This parameter represents the amplitude of the motion. It is defined in terms of observed spectral parameters $S = (Amax - Amin)/[Azz - \frac{1}{2}(Axx + Ayy)]$ when Azz, Axx, Ayy = hyperfine tensors; Amax = ½ of the distance between the outermost lines, and Amin = ½ of the distance from the first minimum to the last maximum of the spectrum for a randomly oriented sample.
- τc = rotational correlation time, used for isotropic motion. τc is obtained from the Kivelson (4) equation:

$$\tau c = 6.5.10^{-10}\ \Delta Ho\ [\sqrt{(Io/I_{-1})} - 1]$$

where ΔHo = width of the central line and I_{-1}, Io = amplitudes of the central and high field lines, respectively.

This work was aimed at evaluating the interest of the spin probe ESR method in the field of fat physics.

In parallel, other methods have been used: differential scanning calorimetry (DSC), NMR and rheological methods. Three fats have been compared: lard and the solid and liquid fractions obtained by centrifugation of lard at 15 C.

METHODS

Pork back fat adipose tissue was ground, then melted for 30 min at 90 C. Lard was obtained by filtration and stored at −30 C. Dry fractionation was performed in a tempered centrifuge (Sorvall, Super Speed RC2). After 3 hr centrifugation (39400 g) at 15 C, an oil phase, so-called 15 C liquid fraction (45% in weight) and a solid fraction (55% in weight) were obtained.

The triglycerides were purified by chromatography on silicic acid. Their composition was determined by means of high pressure liquid chromatography (HPLC) (5).

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Differential scanning calorimetry has been performed using a Perkin-Elmer DSC-2 calorimeter; 20 mg of the sample were placed in aluminium pans (Dupont Instruments). An empty pan was used as a reference. Before cooling, the fat was completely molten (30 min at a temperature 20 C higher than the final melting temperature). Cooling rate was 10 C/min. The sample was maintained for 15 min at −70 C, then heated at a rate of 5 C/min.

Viscosity measurements were performed using a coaxial rotational viscosimeter (Rheomat 30 - Contraves A.G.) associated with a programmer (Rheoscan 20 - Contraves A.G.).

Pulsed NMR (Minispec, 20 - Brucker. Measurements performed at the Laboratoire d'Organisation Moléculaire et Macromoléculaire, CNRS, Thiais, France) was used to determine the solid fat index.

The nitroxide probe was the 16 Doxyl stearic acid (Synvar). Two mg of this radical was dissolved in 3 cm^3 of molten fat, then the sample was inserted in 4 mm internal diameter quartz tubes placed first for 2 hr in a freezing apparatus at −30 C, and then ½ hr in liquid nitrogen.

Finally the sample was inserted in the precooled cavity (−100 C) of the ESR spectrometer (Varian E9). The sample temperature was varied between −50 and +70 C, by 3 C increment increases. A Varian variable temperature control unit calibrated with a Copper-Constantan thermocouple monitored the temperature.

RESULTS

The solid fraction contained more trisaturated triglycerides than the other two fats, and the liquid fraction contained more unsaturated triglycerides (Table 1).

DSC curves (Fig. 1) of lard and solid fraction were very close to each other. Their melting ranges spread from −23 to +54 C. However, the proportion of low melting point crystals was lower in the solid fraction.

For the liquid fraction, thermal phenomena began at −36 C, with an exothermic accident probably due to a post-crystallization of the glycerides which remained in a supercooled state during cooling. Melting was presumed to begin near −23 C as in the other two samples, and it ended at +31 C.

Following an extensive discussion of the results obtained by thermal analysis, we concluded (6) that crystals which melted below 0 C might be induced by triunsaturated glycerides. For lard and the solid fraction, peak A has been attributed to the fusion of crystals induced by diunsaturated, peak B to disaturated and peak C to trisaturated glycerides.

For the three fats, a decrease in the solid fat content, determined by NMR, was observed to begin above −12 C. At 0 C, about 15% of the crystalline fat (lard or fraction) was molten.

Newtonian viscosity has been calculated using the equation: $\sigma = \eta_N D$.

Plastic viscosity has been obtained from the modified Casson equation: $\sigma^{1/2} = 2f^{1/2}/(1 + a) + \eta_p^{1/2} D^{1/2}$, where σ = shear stress; D = shear rate; f = yield value, and a = radius ratio of the inner and outer cylinder.

The Arrhenius-Andrade plot for the variations of the plastic and Newtonian viscosity is shown in Figure 2. No difference could be observed between the three molten samples. The evolution of the ESR spectra with temperature was similar for the three samples.

Figure 3 illustrates the changes in the ESR spectra of lard with temperature:

- at low temperature (T < −30 C) a powder spectrum characteristic of slow tumbling radicals ($\tau c > 10^{-8}$ s) was observed;
- above −2,5 C, a spectrum with three narrow lines, representing the mobile probes ($\tau c < 10^{-8}$) was observed:
- between −30 and −2,5 C, these two kinds of spectra superimposed.

So, within the temperature range studied, two populations of probes could be distinguished: the probes with slow motion, i.e., in a high viscosity environment, and the mobile probes, in a low viscosity medium, probably a liquid phase.

The mobilization process of the probes as temperature increased was evaluated from the i/j parameter which was assumed to vary as the proportion of immobilized probes, where i = amplitude of the low field line of the powder spectrum and j = height of the central line.

For lard this process began at −30 C (Fig. 4). Two ranges could be distinguished:

- From −30 to −12 C i/j decreased moderately
- From −12 to −2,5 C the effect of temperature on the transition: "immobilized"→

TABLE 1

Triglyceridic Composition of Lard and Fractions (% moles) (from 5)

	000	001	002	011	012	111
Solid fraction	8.7	45.6	5.6	30.8	4.9	4.3
Lard	4.7	35.6	5.6	43.5	5.4	5.2
Liquid fraction	2.0	25.7	3.9	55.3	7.2	5.9

0: saturated fatty acid; 1: mono-unsaturated fatty acid; 2: di-unsaturated fatty acid.

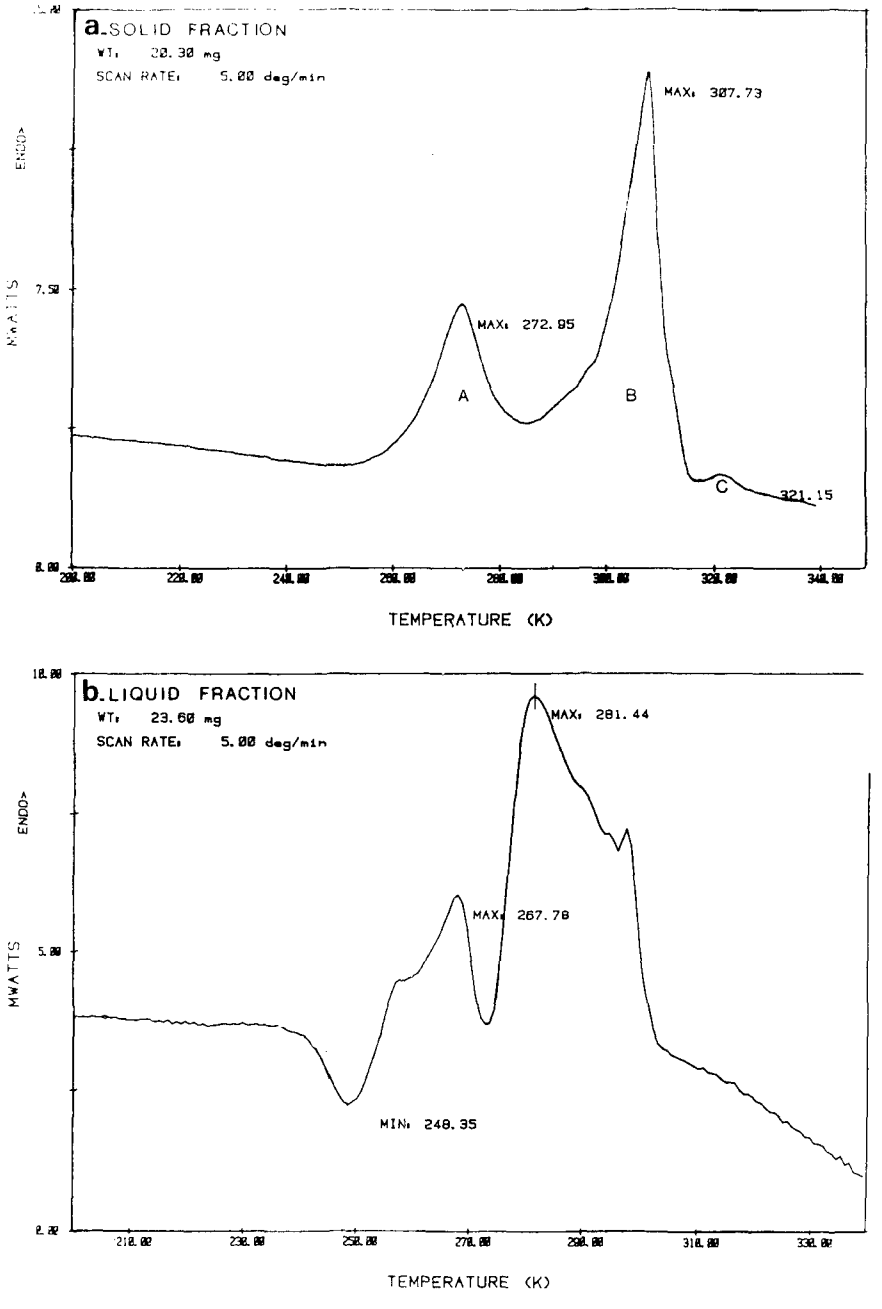

FIG. 1. DSC curves for the solid (a) and liquid (b) fractions.

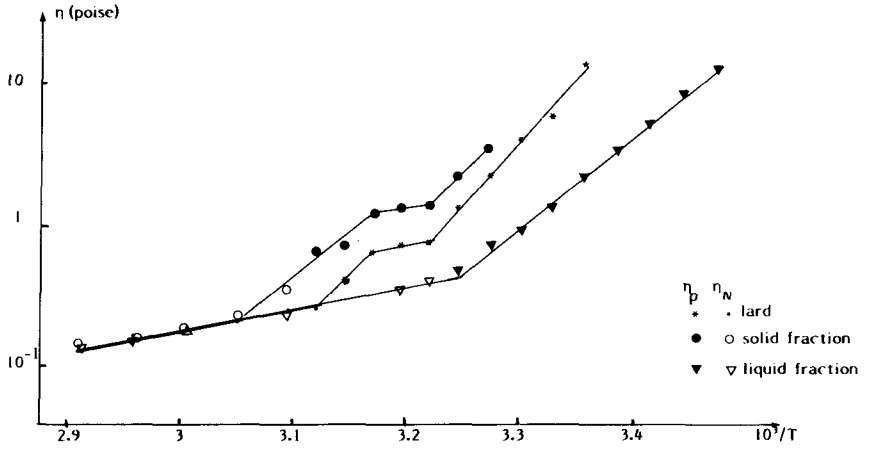

FIG. 2. Arrhenius plot of the variations of the Plastic viscosity (ηp) and Newtonian viscosity (η_N) for lard and for its fractions.

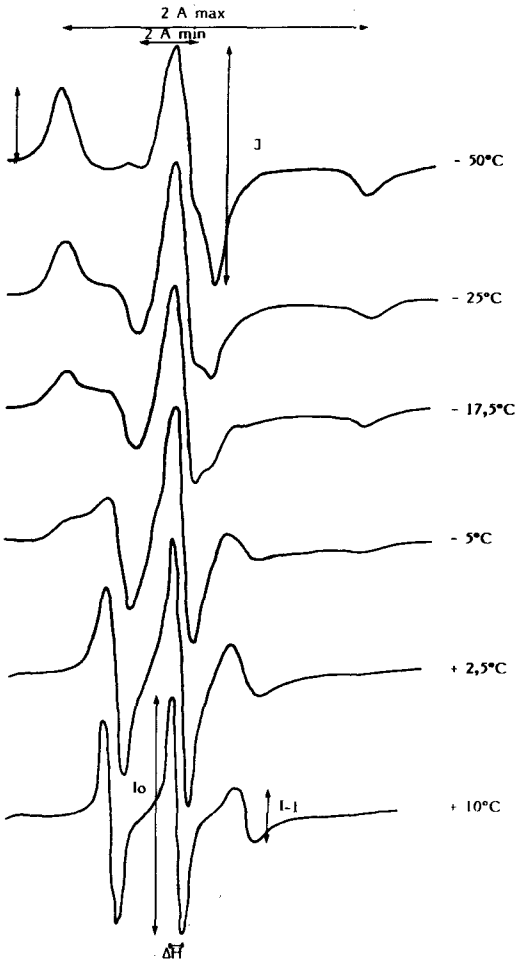

FIG. 3. ESR spectra of the nitroxide probe in lard at different temperatures.

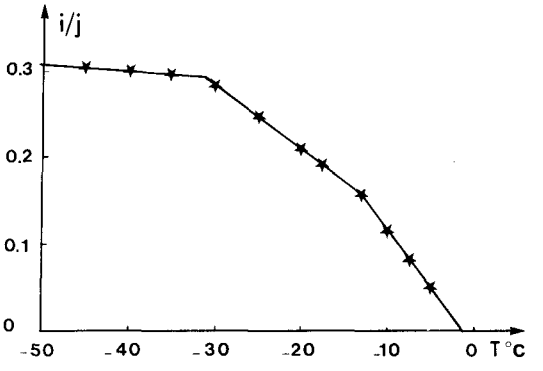

FIG. 4. Change in the proportion of probes with slow motion in lard, in function of temperature.

mobile was more pronounced.

At −2,5 C, the heterogeneity in the environment around the probe was no longer observed; all the probes had rapid motion.

For the solid and liquid fractions, the mobilization process began at −30 C. It ended at −5 C for the liquid fraction and +2,5 C for the solid one. Two ranges also could be distinguished with a transition at −20 C for the two fractions. In comparison with lard, when temperature increased the rate of the mobilization process was higher for the liquid fraction and lower for the solid one.

Mobility of the probes with slow motion = changes in the order parameter S (Fig. 5) indicated that the amplitude of the molecular motion increased with temperature. Moderate between −50 and −30 C, this effect was more pronounced above −30 C. The same evolution was observed for lard and the fractions.

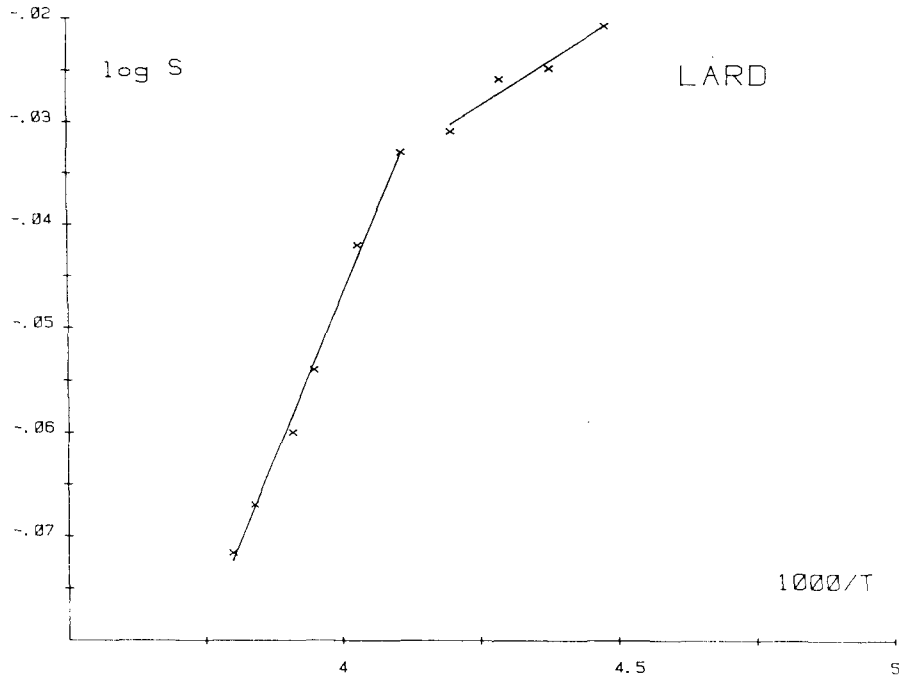

FIG. 5. Arrhenius plot of the order parameter (S), for the fatty acid nitroxide in lard.

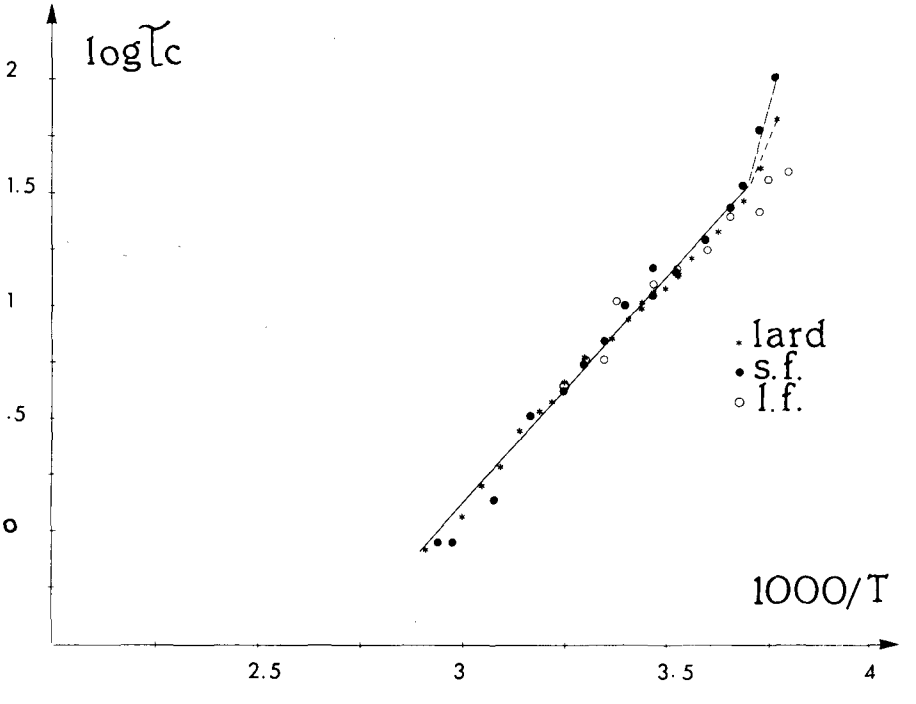

FIG. 6. Arrhenius plot of the correlation time (τc) for the fatty acid nitroxide in lard, in the solid fraction and in the liquid one.

Mobility of the probes in the low viscosity environment = τc decreased when temperature increased. Except at temperatures T < −2,5 C, the probe had the same mobility at the same temperature for the three fats. For lard and the solid fraction, the Arrhenius plot for τc showed two linear portions (Fig. 6). For both samples the activation energies (Ea) corresponding to these portions were similar and close to 9,5 Kcal.mole^{-1} for the higher temperature range. For the lower temperature range, the calculated activation energies were higher 21 Kcal.mole for the solid fraction, 16 Kcal.mole^{-1} for lard.

For the liquid fraction a singular least squares line was observed over all the temperature range studied. Slight differences between probe mobility (τc) in the three fats were observed only at low temperature (Table 2).

TABLE 2

Correlation Time Calculated for Probes in the Three Fats ($\tau c \times 10^{10}$ sec)

	−5 C	+10 C	+30 C
Solid fraction	56.3	13.5	5.3
Lard	41.5	13.4	6.0
Liquid fraction	25	13.9	5.6

DISCUSSION

The probe used for the present ESR study is not a spherical molecule; because of the length of the aliphatic chain some motions are hindered. However, Raison et al. (2) considered that the motion of a spin labelled fatty acid in lipidic media is nearly isotropic. This is more particularly valid when the nitrogen group is far from the carboxyl group of the fatty acid molecule. The order parameter S has been used to follow the changes in the mobility of the fatty acid derivatives with slow motion. S has been calculated only when all the probes had slow motion or when the proportion of free tumbling probes was low. We have used τc to characterize the mobility of the probes with fast motion, i.e. in a liquid environment.

For lard and the solid fraction, in the temperature range −50 to −30 C, no phase change was detected by ESR or DSC. However, although it was very reduced at these temperatures, the amplitude of the motion of the "immobilized" fatty acid derivatives increased slightly with temperature. We can assume that the highly viscous microdomains surrounding the probes are constituted by lipids in a glassy or supercooled state. At −30 C as shown by the Arrhenius plot for S, the mechanism of the mobility increase of the "immobilized" probes changed. This was probably the result of a modification affecting the physical state of these microdomains. At the same temperature, the first mobile probes appeared. We can conclude that a liquid phase was forming in this system, but not detected by DSC (DSC revealed thermal events only above −23 C). The differences in the determination of the beginning of melting with ESR and DSC can be attributed to the fact that ESR is particularly sensitive to phase changes occurring in the neighborhood of the probes, while DSC gives information on the thermal behavior of the bulk fat.

Probe's mobilization resulted from the appearance of a liquid phase which could have different origins: a phase transition of the supercooled lipids or the melting of crystals. The melting behavior of triglyceridic crystals depends essentially upon their chemical composition and polymorphic form. The mobilization process occurred at low temperature and spread over a broad temperature range. So probes seem to be partly associated to crystals induced by unsaturated glycerides (low melting point glycerides) and differing in their composition and/or structure.

Slight differences between the mobility of the mobile probes in the three fats were observed only at low temperature (T < −2,5 C). Above this temperature, no difference was detected although the thermal behavior of these fats differed over the whole temperature range studied. Activation energies calculated from the Arrhenius plot for τc are of the same order as the values given by Mehlhorn et al. (3) for a spin labelled fatty acid dispersed in tripalmitin or elaidic acid within the same temperature range. These authors had observed that the activation energies were only slightly different when the probe was in a solid or a liquid medium.

So, differences between the three samples were observed only when melting phenomena occurred in the neighborhood of the probes. These differences could be attributed to the melting rate in this temperature range.

At temperatures above 0 C, ESR was not ever sensitive to the melting phenomena occurring in the bulk fat. All the probes were dissolved in the liquid phase of the fat; their mobility depended on temperature and not on the composition of this liquid phase, different for the three fats. This result was in accordance with the rheological measurements showing that the three molten fats had the same viscosity at the same temperature.

The Stokes-Einstein equation correlates the

TABLE 3

Values for the Ratio $\tau c/\eta$ for the Fatty Acid Nitroxide in Lard at Different Temperatures and Corresponding Solid Contents

	T C	$\tau c/\eta$[a]	Solid content (%)[b]
Newtonian behavior	70	6.3	0
	60	6.1	0
	55	7.5	0
	50	7.7	0
Viscoplastic behavior	45	6.8	0.5
	40	4.7	2
	35	3.4	6
	30	1.5	18
	25	0.5	25
	20	0.08	30

[a]Determined by viscosimetry (7-8).

[b]Determined by NMR (6).

correlation time (τc) for the nitroxide probe and the viscosity (η) of the medium: $\tau c = 4\pi\eta r^3/3KT$, where r = radius of the studied molecule.

Measurements of the viscosity of lard at different temperatures showed that the ratio $\tau c/\eta$ was nearly constant when the fat, completely molten, exhibited a Newtonian behavior. The ratio showed large variations when crystals were present (Table 3). These results confirm that the changes in τc corresponded to changes in the viscosity of the liquid phase, where probes were dissolved, and not to the changes in the viscosity of the bulk fat.

The Stokes-Einstein equation applies when the medium is homogeneous, that is, when the fat is completely molten.

CONCLUSION

It is generally admitted that fatty acid nitroxide probes behave like impurities when dispersed in lipidic systems. We can conclude from our results that these probes seem partly embedded in amorphous microdomains, and partly associated with low melting point crystals. When all the probes are in the liquid phase, their mobility reflects the viscosity of their liquid environment when crystals are still present and the viscosity of the bulk fat when the fat is completely molten.

Above 0 C, ESR did not permit observation of changes induced by the melting of the major triglyceridic crystals as detected by DSC. Repeated analysis would be necessary to reveal, eventually, more restricted steps corresponding to these phenomena.

We can consider that the behavior of the fatty acid probe gives a good approximation of the behavior of other molecules existing at low concentrations in fat, for example fatty acids liberated during lipolysis.

To study the physical state of a fat by ESR, it would be more interesting to use a triglyceridic nitroxide as probe, i.e. a molecule with a greater probability of being included in the crystalline network. In that case the probe would permit following the behavior of one triglyceridic group in more detail and also be sensitive to the other phase changes occurring in fat.

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METHODS

Analysis of Deuterium Labeled Blood Lipids by Chemical Ionization Mass Spectrometry

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ABSTRACT

A quantitative analytical method has been developed to analyze methyl esters of blood fatty acids derived from human subjects fed deuterium-labeled fats. The GCMS computer method provides for the analysis of the fed deuterium-labeled fatty acids, the naturally occurring blood fatty acids and new fatty acids formed by chain elongation or shortening of the fed labeled fats. Approximately 20 fatty acids including 16, 17, 18 and 20 carbon chain acids were analyzed with a relative standard deviation of 0.02 at the microgram level and a sensitivity of less than one nanogram. The method uses capillary GC to separate the fatty acid esters and isobutane chemical ionization mass spectrometry with multiple ion detection to determine the isotopic constituents of the GC peaks. The technique provides for the determination of overlapping GC peaks labeled with 2, 4 and 6 deuterium atoms and makes extensive use of computers both for data acquisition and processing.
Lipids 20:303-311, 1985.

INTRODUCTION

A gas chromatographic-mass spectrometric technique was developed to analyze fatty acids extracted from the blood of human subjects fed deuterium labeled fats. The technique makes extensive use of standards and computer programming to quantitatively measure the complex mixture of fatty acids. The feeding studies were conducted to determine the metabolic fate of geometrical and positional isomers of fats formed during the commercial hydrogenation of soybean oil. In the metabolism experiments described elsewhere (1-5), monounsaturated fatty acids labeled with 2, 4, and 6 deuterium atoms were fed as a single pulse to human subjects, and the level of labeled and unlabeled fatty acids in the subject's blood was followed with time.

In the previously reported method, only the monoene 18 carbon fatty acids were analyzed (6). In this work the data collection and processing has been expanded to include all of the fatty acids that elute with and have mass numbers in the range of the C16, C17, C18 and C20 fatty acids. This analysis of all the major fatty acids in the blood lipid fractions makes it possible to follow the effects of the pulse of fat in the blood stream as the labeled material is digested, to feed a wider variety of labeled fats and to observe if new fats are being formed by chain elongation or shortening of the labeled fats.

Nine blood samples were drawn from each subject, and each sample was then separated into as many as 40 blood lipid fractions. Each blood fraction contained more than 20 fatty acids, either naturally occurring or labeled. Thus a total of more than 7,200 mass peaks must be identified and measured per subject. To handle the large number of mass peaks, a computer program has been developed to identify, measure and compute the various components present in the mixture.

EXPERIMENTAL

The labeled fatty acids, *trans*-10-octadecenoic-14,15-d_2 acid, 18:1-d_2, *cis*-10-octadecenoate-14,14,15,15-d_4 acid, 18:1-d_4, and *cis*-9-octadecenoate-14,14,15,15,17,18-d_6 acid, 18:1-d_6, were prepared by coupling the appropriate deuterium labeled alkyl halides and aldehydic esters by the Wittig reaction (7,8). Triglycerides of the labeled fatty esters were prepared by heating with toluenesulfonic acid and glycerol (9).

Feeding of the deuterium labeled triglyceride mixture followed the general protocol previously described (4,5). The mixtures of fatty acids, converted to triglycerides, were fed (9 g of each triglyceride, 27 g total) to subjects as a single pulse. Sequential blood samples were obtained at 0, 2, 4, 6, 8, 12, 15, 24 and 48 hr.

The blood plasma was extracted by the Folch procedure (2:1 $CHCl_3$:MeOH) (10). The plasma samples were isolated by preparative TLC by standard methods, which involved first developing the plates with 6:8:8:1 PE:$CHCl_3$:CH_3OH:H_2O (11) to separate phospholipid classes,

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followed by rechromatographing the neutral lipids with benzene:hexane (15:85) (12). Known weights of 17:0 internal standard were added to the plasma lipids after TLC separation and before conversion to the methyl esters. The separated lipid classes were esterified with HCl-MeOH (13).

A weighed standard containing 11 methyl esters was prepared to provide equivalent chain length (ECL) and mass spectrometer sensitivity calibration for the method. The mixture containing methyl palmitoleate (16:1), methyl palmitate (16:0), methyl heptadecanoate (17:0), methyl linoleate (18:2), methyl oleate (18:1), methyl stearate (18:0), methyl arachidonate (20:4), methyl eicosatrienoate (20:3), methyl eicosadienoate (20:2), methyl eicosenoate (20:1) and methyl arachidate (20:0), was prepared from high purity methyl esters.

One microliter of an isooctane solution containing 1 μg of each ester was injected into a 30 M, wide bore (0.31 mm) thick film (1 μM) DB 1 bonded phase, fused silica column. The exit end of the column was positioned within 20 cm of the mass spectrometer electron beam with the entire GC effluent conducted into the ion source. The capillary GC was operated with helium as the carrier gas with a linear velocity of 87.5 cm/sec, a 1:1 input split ratio and temperature programmed from 176 C to 221 C at 2.3 degrees per min.

The gas chromatograph-mass spectrometer system was a Finnigan Model 4000 with a Finnigan INCOS 2000 data system. The chemical ionization spectra of all samples were obtained with isobutane as the reagent gas to maximize the molecular ion region of the mass spectrum and to reduce the amount of fragmentation both of the samples and of any background materials present. The isobutane pressure of 0.2 Torr was measured by the Finnigan source thermocouple gauge, and the source temperature was held at 140 C. It was observed that at higher reagent gas pressures (0.4 Torr) partial hydrogenation of the unsaturated fatty acids occurred (14). The 11 components of the methyl ester standard mixture were completely separated by the GC column. The intensity values of the molecular ion regions were measured with a four-stage INCOS multiple-ion-detection (MID) descriptor which measured odd-numbered mass intensities only, covering the 16:1 and 16:0 from mass 269 to mass 279; 17:0 from mass 283 to mass 293; 18:2, 18:1 and 18:0 from mass 295 to mass 305 and 20:4 to 20:0 from mass 319 to mass 331. Figure 1 is an INCOS computer map produced after analysis of the standard mixture by GCMS using the data acquisition program. The computer map shows in diagrammatic form the MID descriptors along with the GC separation of the 11 component standard mixture.

In general the GC provided complete separation of the methyl esters, with the exception of

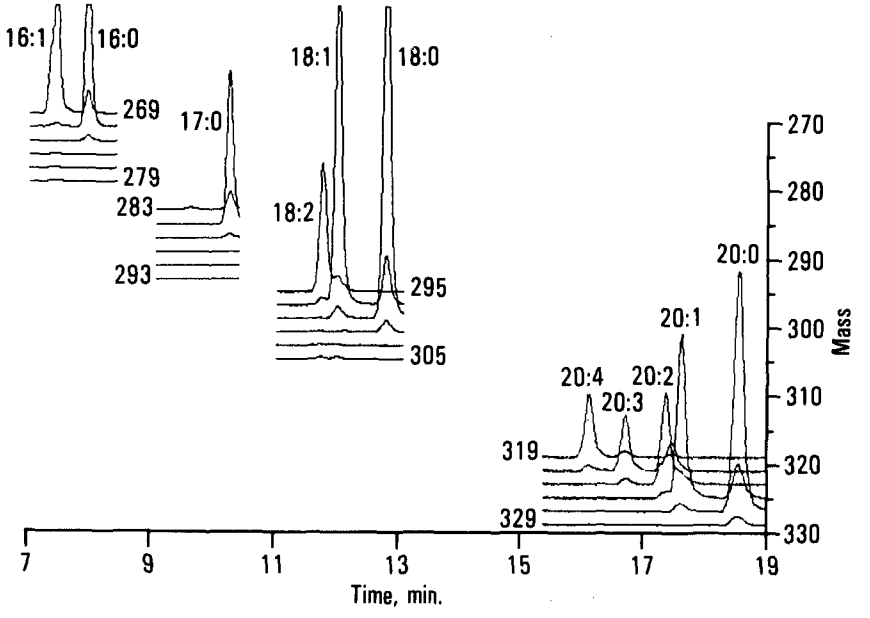


FIG. 1. Mass chromatograms of 11 component blood lipids standard mixture acquired by a four-phase multiple-ion-monitoring technique.

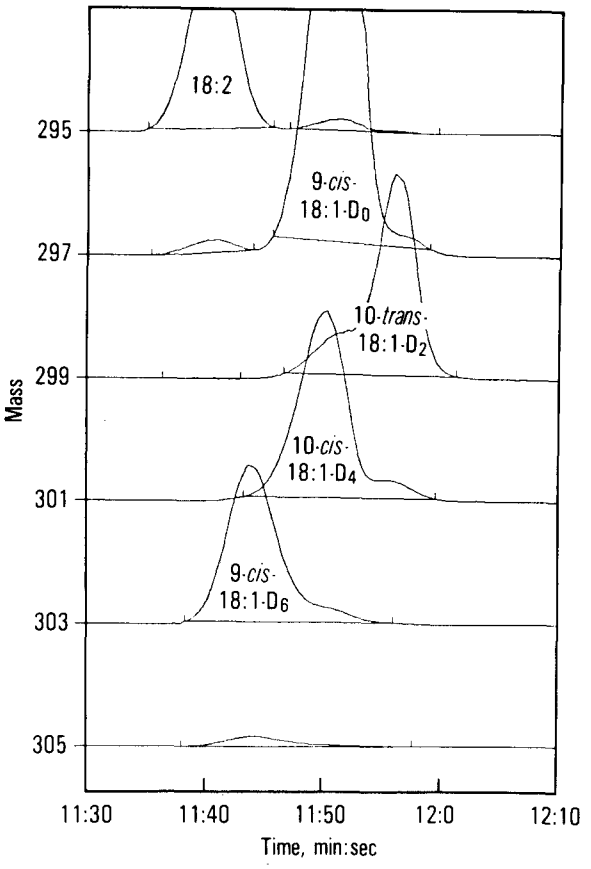


FIG. 2. Mass map of C18 monoene region of the mass chromatogram of 50% labeled fed mixture sample. Sample contains 25% 18:2, 40% 9-*cis*-18:1-d_0, 10% 10-*trans*-18:1-d_2, 13% 10-*cis*-18:1-d_4, and 12% 9-*cis*-18:1-d_6.

the labeled compounds. The d_6 labeled ester eluted about a half peak width before the unlabeled ester, and the *trans* isomer eluted about one-third of a peak width after the unlabeled ester. Figure 2 is the mass chromatogram of the GC monoene region of a 50% label sample made by adding an equal weight of methyl oleate to the methyl esters derived from the labeled fed mixture. Methyl linoleate was also added to the 50% label mixture to further test the separation power of the GC. The naturally occurring 9-*cis*-18:1, the labeled 10-*trans*-18:1-d_2, 10-*cis*-18:1-d_4 and the labeled internal control, 9-*cis*-18:1-d_6, are only partially resolved by the GC, and their spectra interfere with each other. The M+2 peak of one compound overlays the mass and time region of the next higher mass. A computer program has been written to correct for these interferences. A nonpolar GC column was chosen for this work so that peaks would elute in the order diene, monoene, saturate and the higher mass compounds would not tail into the lower mass compounds and interfere at the critical M+2 mass peak.

The analytical procedure, outlined in Figure 3, shows the various data processing steps. The data was collected on a Finnigan INCOS computer system using the MID data collection program. The data was stored as Time/Intensity Values on the INCOS disk. An overnight batch program processed the Time/Intensity data into Mass/Intensity data using Fortran programming on the Finnigan computer. The data was transmitted 500 ft over a 16 bit parallel link to a Modcomp Classic Computer where it was stored on disk and magnetic tape. Another program automatically calculated the area under the mass chromatogram peaks and subtracted background. This program is different from commercial programs in that area associated

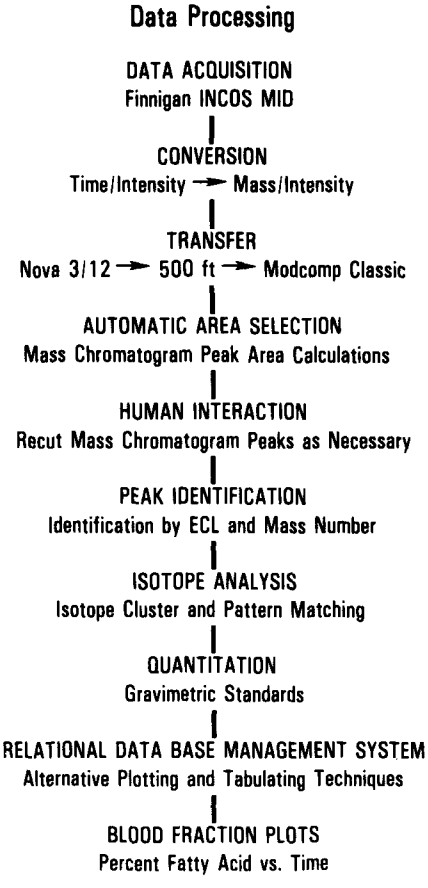
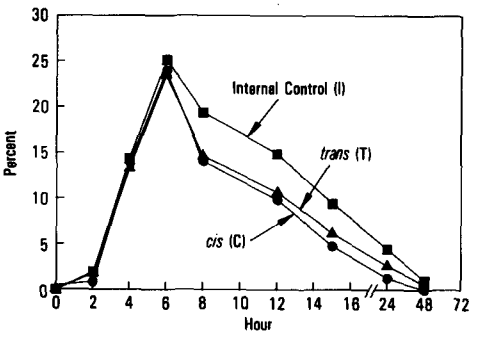


FIG. 3. Outline of blood lipids data processing scheme.



Hour	%D0-N	%D2-C	%D4-T	%D6-I	%Label
0	99.54	0.46	0.00	0.00	0.46
2	95.50	0.83	1.76	1.91	4.50
4	58.54	13.84	13.27	14.35	41.46
6	27.50	23.93	23.45	25.12	72.50
8	52.01	14.05	14.60	19.33	47.98
12	64.74	9.79	10.63	14.83	35.25
15	79.71	4.75	6.15	9.39	20.29
24	91.67	1.25	2.61	4.47	8.33
48	98.48	0.00	0.55	0.98	1.53

	Selectivities			Log-Selectivities		
Hour	C/I	T/I	T/C	C/I	T/I	T/C
0	100.00	100.00	−100.00	100.00	100.00	−100.00
2	−2.50	−1.15	2.17	−0.40	−0.06	0.34
4	−1.13	−1.15	−1.20	−0.05	−0.06	−0.01
6	−1.14	−1.14	1.00	−0.06	−0.06	0.00
8	−1.49	−1.40	1.06	−0.17	−0.15	0.03
12	−1.65	−1.48	1.11	−0.22	−0.17	0.05
15	−2.15	−1.62	1.33	−0.33	−0.21	0.12
24	−3.88	−1.82	2.14	−0.59	−0.26	0.33
48	−100.00	−1.89	100.00	−100.00	−0.28	100.00

Area Selectivities			Area Log-Selectivities		
C/I	T/I	T/C	C/I	T/I	T/C
−1.79	−1.45	1.23	−0.25	−0.16	0.09

FIG. 4. Typical output plot of the fed deuterium labeled fatty acids with tables of selectivities. Percent plasma triglycerides vs hr. The letters refer to N, naturally occurring; C, *cis*; T, *trans*, and I, internal control.

with each mass under each chromatograph peak is stored separately for future use in isotope analysis calculations, rather than have all masses under a GC peak summed. At this point in the data processing, the operator may make adjustments in the way the mass chromatogram peaks are cut to correct for peak shapes that are beyond the capabilities of the computer's algorithm. The data is available at several Tektronix 4010 display terminals where the data can be displayed as a series of mass maps, Figure 2, plotting intensities of the GC curve at each mass number vs scan number or time. The computer-chosen cuts determining the areas under the mass chromatogram are displayed as short vertical marks on each side of the GC peaks, and the operator can either accept the computer's cuts or change them.

The mass chromatogram peaks were automatically identified using equivalent chain lengths and mass number. The isotope distribution patterns for the 11 standard compounds and the labeled compounds were measured and stored in the Modcomp computer. The computer set up a series of simultaneous equations for the mass peaks at each GC peak, and used the isotope distribution patterns to solve the equations for the isotope distribution of the compounds by a least squares method. The measured intensities of the weighed diene standard mixture were used to determine the mass spectral sensitivities of the methyl fatty esters. The intensities assigned to each compound by the isotope distribution analysis program were multiplied by the mass spectral sensitivity for the respective methyl ester to convert the data to micrograms of each ester entering the mass spectrometer. When the samples were processed chemically, a measured weight of internal standard, 17:0 fatty acid, was added prior to sample methylation. The mass spectrally determined

weight of the 17:0 was set equal to the weight of internal standard added and the rest of the ester weights were adjusted proportionally, yielding a set of weight values which represent the weight of each fatty acid in the bottles at the time the 17:0 internal standard was added. The weights of identified peaks were stored in a relational data base system so that the data could be processed in many ways. Plots of intensity vs time, similar to Figures 4 and 7, for each blood fraction were generated.

RESULTS

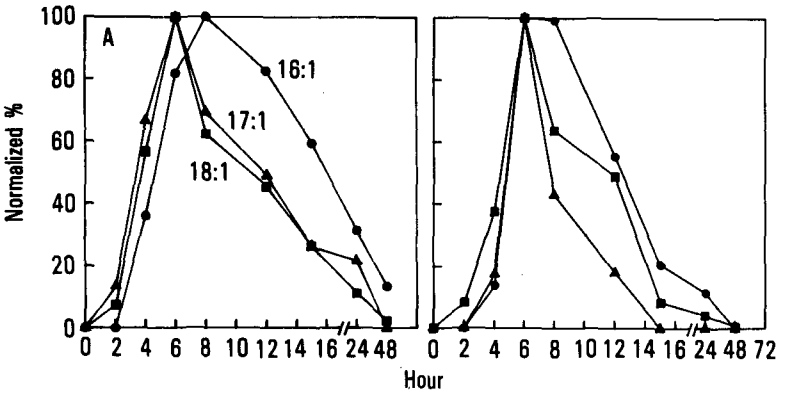
The output from the gas chromatograph-mass spectrometer-computer system is a summary table for each sample injected into the mass spectrometer, listing names of identified compounds, their mass numbers, ECL values, the total intensity counts and the weight of each component in the sample at the time the internal standard was added. The data can be combined into tables and expressed as micrograms, percent of fatty acid or micrograms of fat per ml of the subject's blood. Table 1 contains all of the reportable data for the blood plasma triglyceride fraction for the nine hourly samples. It shows the fatty acids normally found in blood and the deuterium labeled components of the 16, 17 and 18 carbon monoene fatty acids. The main computer program will process, identify if possible and measure all mass peaks collected by the multiple-ion-detection system of the mass spectrometer. Only a very few mass peaks were unidentified, and these were small (summed to less than 1%) and had essentially the same intensity regardless of hour. These were probably nonfatty ester materials extracted from the blood.

The purpose of the metabolism experiment was to determine if the human body is selective in its handling of the different fatty acid isomers. The three labeled C18 monoene fatty esters were plotted as percent of total C18 monoene to emphasize the changes in the fed material as it passes through the subject's body. This plot, plus the monoene percent data, the selectivities and the area selectivities are a standard computer output as shown in Figure 4. To determine if the human body discriminates for or against the fed isomers, it is helpful to take the ratios of various combinations of isomers. The internal control 9-*cis*-18:1-d_6, "I", was fed to all subjects and the ratio of 10-*cis*-18:1-d_4, "C", to the internal control, C/I, and the ratio of 10-*trans*-18:1-d_2, "T", to the internal control, T/I, both provide a measure of discrimination which is comparable among subjects. Similarly the ratio of *trans* to *cis*, T/C, provides a measure of the discrimination for or against the *trans* isomer as compared to the *cis* isomer. Since these ratios would be affected by the ratio of the materials fed to the subject, the selectivity is defined as the ratio of the isomers found in the blood divided by the ratio of the same set of isomers that were fed.

TABLE 1

Plasma Triglycerides, Micrograms of Lipid per Milliliter of Blood Plasma

	Hours								
Sample	0 hr	2 hr	4 hr	6 hr	8 hr	12 hr	15 hr	24 hr	48 hr
16:1 D0	7.04	12.48	9.28	14.26	9.88	15.66	18.11	19.23	18.31
16:1 D2	0.40	0.32	0.94	2.27	1.78	1.87	1.29	0.67	0.52
16:1 D4	0.00	0.00	0.56	2.28	1.99	2.37	1.79	0.92	0.36
16:1 D6	0.00	0.00	0.30	1.17	0.61	0.71	0.42	0.20	0.00
16:0	76.46	68.23	75.70	98.88	62.80	95.83	122.63	104.75	116.18
17:1 D0	1.15	1.43	1.39	1.88	1.24	1.96	3.11	2.27	1.73
17:1 D2	0.52	0.43	0.86	2.85	0.96	1.22	2.12	1.05	0.64
17:1 D4	0.00	0.14	1.49	7.27	1.62	1.31	0.89	0.44	0.00
17:1 D6	0.00	0.03	0.60	2.03	0.70	0.68	0.53	0.23	0.00
18:2	41.73	64.08	73.35	121.34	66.51	78.90	83.93	87.53	125.75
18:1 D0	96.76	127.30	150.58	221.07	137.69	173.55	239.64	209.93	256.48
18:1 D2	0.45	1.10	35.75	193.28	37.14	26.31	14.23	2.85	0.00
18:1 D4	0.00	2.34	34.18	189.49	38.66	28.55	18.49	5.98	1.42
18:1 D6	0.00	2.53	37.06	202.12	51.16	39.86	28.22	10.23	2.55
18:0	22.03	15.20	19.68	27.56	16.88	22.20	45.71	18.69	19.28
20:4	3.17	4.76	5.13	7.94	5.03	4.51	4.86	3.67	6.56
20:3	0.62	1.10	0.95	1.57	1.05	0.88	1.06	1.17	1.55
20:2	0.55	0.67	0.77	1.06	0.66	0.84	0.73	0.83	1.20
20:1	0.76	0.88	0.85	1.29	0.86	0.89	1.59	1.17	1.27
20:0	0.64	0.46	0.87	0.55	0.41	0.41	0.78	0.46	0.34
Sum	251.98	303.48	450.29	1,100.20	437.62	498.54	590.14	472.27	554.14



Full Scale Represents

	(A) TG			(B) FFA		
	16:1-D_4	17:1-D_4	18:1-D_4	16:1-D_4	17:1-D_4	18:1-D_4
μg into MS	0.015	0.06	1.5	0.007	0.04	1.25
μg Total Separated Fraction	12.500	45.40	1182.0	0.600	3.00	101.70
μg/ml in Blood	2.000	7.30	189.0	0.090	0.46	15.50

FIG. 5. Plot of 18:1-d_4, 17:1-d_4 and 16:1-d_4 to demonstrate sensitivity of the method. A) Plasma triglyceride-d_4. B) Plasma free fatty acid-d_4.

$$\text{Selectivity} = \frac{\text{isomer (blood fraction)/oleic analog (blood fraction)}}{\text{isomer (fed)/oleic analog (fed)}}$$

The ratios are not symmetrical across "1". The ratio of a pair with 20% of one compound and 80% of the other compound is either 4 or 0.25, depending on which is the numerator. This makes interpretation difficult. To make the selectivity symmetrical across "1", a computer program has been written to test if the ratio is less than 1 and, if so, to take the reciprocal of it and multiply by minus 1. These values are shown as selectivities in Figure 4. Another way to make the selectivities symmetrical is to take the logarithm of the ratio, which makes the value symmetrical across "O". These values are shown as log-selectivities in Figure 4. In both cases, selectivities greater than 100 are set to 100 as an upper limit. Also of interest are areas selectivities, where the areas under the curves are used in the selectivity calculations.

During preparation of the 10-*trans*-18:1-d_4, 5% of 17:1-d_4 and 0.5% of 16:1-d_4 also were formed and were included in the fatty acids fed to the human subject. Figures 5, A and B are plots of plasma-TG and FFA blood fractions containing these tetradeuterated fatty acids. These plots demonstrate the greatly improved sensitivity of the present method. The data have been normalized, with the largest peak in each fraction set to 100 so that the data can be compared. The actual weights these plots represent are shown on the adjacent table. The 17:1 curve follows the 18:1 closely, and the weight of the maximum intensity at 6 hr is proportional to the 5% found in the fed mixture. The 16:1-d_4, on the other hand, has a much broader curve delayed slightly from the 18:1-d_4; moreover, the most intense peak is twice as large as it should be compared to the amount of 16:1-d_4 in the fed mixture. If the area under the curves is considered, there is more than four times as much 16:1-d_4 as would be expected. This means that the body is converting some 18:1-d_4 to 16:1-d_4 or is specifically accumulating 16:1-d_4 in the blood. The curves in Figure 5A were produced from the equivalent of 0.1 ml of the subject's blood, and the curves in Figure 5B were produced from the equivalent of 1 ml of blood.

Figure 6 is a small section of the mass chromatogram of 16:1-d_4 at mass 273, which corresponds to the point at 4 hr and 14% normalized on Figure 5B. The mass chromatogram has been expanded 2,000 times to show the size of the peak in relationship to the background noise. This peak, with a signal-to-noise ratio of about 5, represents 1 nanogram of methyl ester. The peaks and background are all measured in terms of areas, which reduced the effects of the background noise.

FIG. 6. Mass chromatogram of small peak of 16:1-d_4 at mass 273 with a signal-to-noise ratio of 5 for 1 nanogram of methyl ester.

TABLE 2

Precision of Measurement of 11 Fatty Acid Mixtures

Sample number	Fatty acid methyl ester										
	16:1	16:0	17:0	18:2	18:1	18:0	20:4	20:3	20:2	20:1	20:0
1	12.64	11.58	13.02	7.10	9.57	9.68	5.60	4.22	7.43	8.17	10.98
2	12.65	11.66	13.04	7.05	9.55	9.75	5.54	4.15	7.38	8.16	11.06
3	12.54	11.10	12.81	7.03	9.76	9.85	5.71	4.22	7.39	8.38	11.21
4	12.66	11.65	13.03	7.17	9.59	9.70	5.49	4.11	7.41	8.17	11.01
5	12.68	11.59	12.97	7.09	9.58	9.66	5.56	4.16	7.45	8.26	11.00
6	12.86	10.92	12.29	7.31	9.63	9.38	5.81	4.35	7.89	8.49	11.08
7	12.77	11.50	13.19	7.03	9.64	9.85	5.51	4.12	7.34	8.08	10.97
8	12.17	11.48	13.58	7.20	10.01	10.20	5.30	3.96	7.05	7.94	11.03
9	12.70	11.45	12.85	7.26	9.74	9.78	5.69	4.23	7.06	8.15	11.10
Mean	12.63	11.44	12.98	7.14	9.67	9.76	5.58	4.17	7.38	8.20	11.05
s	0.194	0.256	0.342	0.103	0.146	0.216	0.148	0.107	0.245	0.161	0.075
% RSD	1.54	2.24	2.63	1.44	1.51	2.22	2.66	2.57	3.32	1.97	0.68

Average % RSD = 2.07.

TABLE 3

Precision of Measurement of 50% Fed Labeled Sample

Sample number	Fatty acid methyl ester			
	18:1	18:1-D2	18:1-D4	18:1-D6
1	53.05	13.57	17.41	15.97
2	52.94	13.55	17.46	16.05
3	53.02	13.45	17.47	16.06
4	53.13	13.39	17.44	16.04
5	53.21	13.34	17.44	16.02
6	53.21	13.36	17.43	16.00
7	53.27	13.29	17.42	16.02
8	53.31	13.27	17.41	16.00
Mean	53.14	13.40	17.44	16.02
s	.13	.11	.02	.03
% RSD	.24	.84	.13	.19

Average % RSD = .35.

The analytical method described here was used to analyze the blood lipids from a human subject fed *cis*-10 and *trans*-10 deuterium labeled octadecenoic acids. The analyses were conducted over a period of more than 4½ mo. At the beginning of each day, the 11-component methyl ester mixture was run for sensitivity calibration for that day's unknowns. As an experiment to determine the reproducibility of the method, the same 11-component mixture occasionally was run again late in the day. The data for nine runs are shown in Table 2. The data show an average relative standard deviation of 0.020 during the processing of almost 500 samples and many instrument tunings. Table 3 shows the results of 8 analyses of the 50% fed label mixture displayed in Figure 2. The average relative standard deviation was 0.0035.

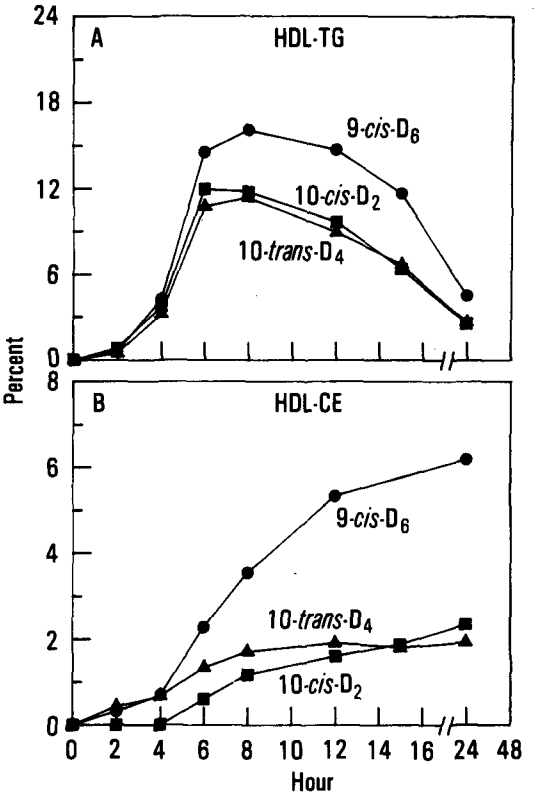


FIG. 7. Typical results of the analysis of fatty acids from a subject fed deuterium labeled lipids. A) High-density lipoprotein triglyceride. B) High-density lipoprotein cholesterol ester.

Typical results for the analysis of blood lipids from a 24-yr-old male subject fed 27 grams total of labeled fatty acids after an overnight fast are shown in Figure 7A, the plot of the high-density lipoprotein triglyceride fraction, and 7B, the plot of the high-density lipoprotein cholesterol ester fraction. Twenty milliliters of blood were drawn at each time increment. The fourth component of the mixture, 18:1-d_0, is not plotted.

DISCUSSION

Feeding mixtures of deuterium labeled fatty acids to human subjects is a very effective method for following the metabolism of fats. The dual-label technique, where the subject is fed a labeled analog of a naturally occurring fat along with the labeled test material, has a great advantage over single-label techniques. The labeled analog is used as internal standard or control, which is subjected to all the normal metabolic processes and cancels out most of the effects of subject to subject variation. Thus, variations in the subject's rate of metabolism for fats affect both the test material and the control equally, and the difference provides a measure of the body's discrimination for or against the test material as compared to the naturally occurring material. This internal standard or control greatly reduces the number of human subjects it is necessary to feed to obtain meaningful results. Our present work uses a triple-label system in which two deuterium labeled isomers are fed along with the deuterium labeled control.

Blood fraction methyl esters present several difficulties to the mass spectroscopist. The molecular ion of the monounsaturated methyl ester is only 1% of the electron impact total ionization. Chemical ionization mass spectrometry overcomes this with a pseudo molecular ion, $M+H^+$, which contains approximately 85% of the total ionization. Chemical ionization causes other problems, complicating the molecular ion cluster with ions such as the molecular ion, the molecular ion minus a hydride, M-1, and adduct ions. So long as the molecular ion cluster can be measured with good reproducibility, correct information can be extracted from the data by the use of standards.

To reduce the difficulty of preparation of the labeled fatty acids and the likelihood of isotope effects in the metabolism experiment, the number of deuterium atoms was limited to 2, 4 and 6. At the molecular weight of the C18 fatty esters, the ^{13}C second isotope peak, M+2, is 2.5% of the molecular ion. This means that if the d_2 labeled compound is 2.5% of the monoene in the blood sample, the interfering M+2 peak for unlabeled monoene, d_0, will be of equal height. Again, good reproducibility of the molecular ion cluster will allow extraction of information by the use of standards. The naturally occurring compounds, linoleate, oleate and stearate, contribute to the complexity of the GC peaks and can interfere with the dideutero ester of the next higher degree of unsaturation. Thus, tailing from methyl stearate, 18:0, interferes with labeled methyl oleate, 18:1-d_2. Tailing varies with size of the sample components and efficiency of the GC column and is difficult to correct. The problem can be avoided by the use of nonpolar columns, where the highest molecular weights elute after the unsaturated lower molecular weights.

The extensive computations done in this method require very reproducible intensity data. The mass marking and mass stability of the mass spectrometer appear to be very dependable over a period of weeks. Absolute intensities and mass discrimination, the variation of sensitivity with changes in mass of the recorded ion, are

very much affected by source conditions and tune of the instrument and vary on a daily basis. To correct for mass discrimination effects, the 11-component methyl ester mixture is analyzed daily to determine current mass sensitivity. The 17:0 internal standard is used to determine absolute sensitivity and to correct for the difficult to control variables such as GC injection efficiency, mass spectrometer multipler gain and source ionization efficiency.

Trans monoenes and monoenes with double bonds in other than the 9 position from commercially hydrogenated fats that the subject acquired in his normal diet were not completely separated by the GC from the methyl oleate peak, and this caused data handling difficulties. This complex mixture of fatty esters appeared as a shoulder on the back side of the methyl oleate peak, with an intensity up to 15% of the oleate peak, and had a peak width 1½ times the oleate peak width. The oleate peak and shoulder were hand cut to include all the monoene in one peak. The resulting broad peak had a higher ECL than the normal 18:1 standard and caused trouble in the software compound identification algorithm, which required that the ECL of the broad peak be entered into the ECL calibration table.

The methods and the computer algorithms used in this work have been developed over a period of years, covering the analysis of samples from 11 subjects. The method described was used on one subject and included complete analysis of 9 blood samples, each separated into 40 blood fractions, with very satisfactory results.

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Preparation of Hydroperoxy and Hydroxy Derivatives of Rat Liver Phosphatidylcholine and Phosphatidylethanolamine

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ABSTRACT

A convenient method for the preparation of hydroperoxy and hydroxy derivatives of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) is described. PC and PE obtained from rat liver were oxidized with singlet oxygen by using methylene blue as the photosensitizer, and their hydroperoxides were isolated with the aid of reverse phase liquid chromatography. The hydroxy derivatives were obtained by reducing the hydroperoxides with sodium borohydride. The results of gas chromatography mass spectrometry revealed that hydroxy fatty acid components of the hydroxy derivatives were derived from isomeric hydroperoxides of oleic acid, linoleic acid, arachidonic acid and docosahexanoic acid. Normal phase high performance liquid chromatography did not separate the hydroperoxy and hydroxy derivatives from the respective unoxidized phospholipids, although unoxidized PC and PE were separated from each other. However, the hydroperoxy and hydroxy derivatives could be distinguished from unoxidized phospholipid species on reversed phase thin layer chromatography.
Lipids 20:312-317, 1985.

INTRODUCTION

It has been suggested that the peroxidation of membrane phospholipids produces a variety of oxidation products involving hydroperoxides (1). This reaction is believed to cause physiological damage in a biological system (2-4). On the other hand, glutathione dependent lipid peroxidation preventing factors including glutathione peroxidase and glutathione-S-transferase have been well documented (5-10). It has been demonstrated that selenium containing glutathione peroxidase purified from bovine red blood cells has no reactivity with hydroperoxidized phospholipids (11). However, Ursini et al. (12) have indicated that a protein purified from pig liver can reduce the hydroperoxide group of phospholipids resulting in hydroxy derivatives at the expense of glutathione oxidation. Preparation of hydroperoxy and hydroxy derivatives of membrane phospholipids seems, therefore, to be required not only for studies on the biological effects of lipid peroxidation products but also for studies on the cellular defense mechanism against lipid peroxidation. Phosphatidylcholine (PC) hydroperoxides have been isolated from peroxidized palmitoyllinoleoyl PC (13), soybean PC (14,15), dilinoleoyl PC (16), and stearoyllinoleoyl PC (17) by reverse phase high performance liquid chromatography (HPLC). In our previous study (18), a reverse phase column was used to separate hydroperoxides from dilinoleoyl PC and soybean PC which were oxidized with singlet oxygen ($^1O_2$).

The purpose of this study is to develop a convenient method for preparing hydroperoxides of membrane phospholipids and their hydroxy derivatives. Two major phospholipids in rat liver, PC and phosphatidylethanolamine (PE), were oxidized with $^1O_2$. Their hydroperoxides were isolated from the oxidized lipids by using reverse phase liquid chromatography, and their hydroxy derivatives were prepared by chemical reduction of their corresponding hydroperoxides.

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EXPERIMENTAL PROCEDURES

Materials

Male Sprague-Dawley rats (6 weeks old) were killed by decapitation and the livers removed, washed with normal saline and kept at -80 C prior to lipid extraction. Total lipids were extracted from the liver by the method described by Bligh and Dyer (19). The phospholipid fraction was obtained from the total lipids by acetone precipitation according to the procedure of Kates (20). PC and PE were isolated from the phospholipid fraction by column chromatography as follows: silica gel 60 (Merck, Darmstadt, 70-230 mesh), activated at 110 C for 2 hr, was thoroughly mixed with chloroform and packed into a column (200 x 20 mm). The phospholipid fraction was charged on the column and eluted with 200 ml each of solutions of methanol in chloroform with 20, 30, 50 and finally 100% methanol. PC and PE were eluted with 100% methanol and 50% methanol solution, respectively. The purities of PC and PE obtained were evaluated by thin layer chromatography (TLC) on silica gel plate (Merck, silica gel 60 F254, 0.25 mm thick) with chloroform/methanol/water/acetic acid (25:15:

2:4, v/v/v/v) as the developing solvent.

Oxidation of Phospholipids and Isolation of Their Hydroperoxides

Photosensitized oxidation of PC and PE and isolation of PC-hydroperoxides and PE-hydroperoxides from the reaction mixtures were carried out by the method described previously (18). PC (50 mg) or PE (50 mg) was dissolved in 5.0 ml of a mixture of methanol and chloroform (1:1, v/v) containing 0.1 mM of methylene blue followed by photoirradiation with a 30 W tungsten projection lamp for 10 hr at 10 C. After the reaction was completed, the reaction mixture was passed through a Pasteur pipette packed with Florisil (100-200 mesh) to remove methylene blue. After evaporation in vacuo, the concentrated reaction mixture was charged onto a glass column (240 x 10 mm) prepacked with Lichroprep RP-8 (Merck, octane binding-silica gel powder, 40-63 μm size) and eluted with a mixture of chloroform/methanol/water (1:10:0.5, v/v/v). Solvent flow was maintained at 1.8 ml/min and 1.0 ml fractions collected. Phosphorus content and absorbance at 235 nm of each fraction were determined as described previously (18). Fractions containing PC-hydroperoxides and PE-hydroperoxides were collected and concentrated in vacuo.

Preparation of Hydroxy Derivatives

A methanol solution (2.0 ml) of phospholipid hydroperoxides was allowed to stand at 4 C for 30 min after addition of a few crystals of $NaBH_4$. Products were extracted with chloroform three times. To purify the hydroxy derivatives, HPLC was run on a column of Zorbax SIL (4.6 x 250 mm, 5 μm in particle size) with a Shimadzu Dupont liquid chromatograph LC-4A. A Shimadzu SPD-2AS variable length UV detector was used to monitor the effluent at 234 nm or 210 nm. The eluting solvent consisted of 5% water in acetonitrile for the first 5 min followed by a 15-min linear gradient increase of water from 5 to 20%. After the linear gradient elution, the water concentration in acetonitrile was held at 20% for 10 min. The solvent flow rate was maintained at 1.0 ml/min. Fractions containing hydroxy derivatives were collected and concentrated in vacuo.

Thin Layer Chromatography

Reverse phase TLC was carried out by using RP-8 F254S HPTLC plate (Merck). The developing solvent was a mixture of chloroform/methanol/water (1:10:0.5, v/v/v). Bands were detected by spraying with 50% sulfuric acid in ethanol followed by heating.

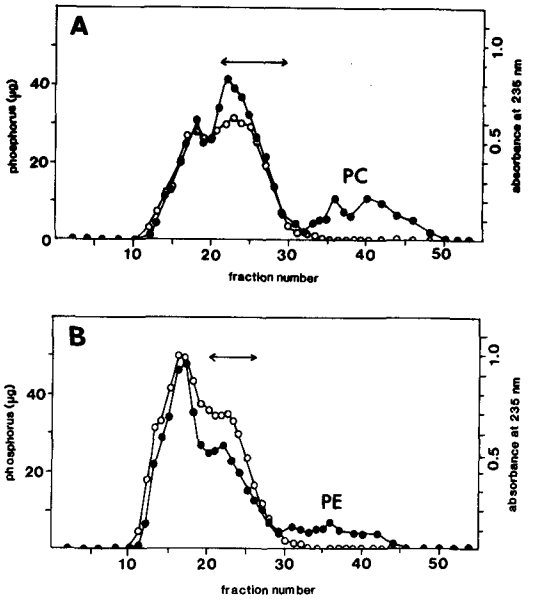

FIG. 1. Reverse phase liquid chromatography of oxidized rat liver PC (A) and PE (B). —●— : phosphorus content; —○— : absorbance at 235 nm after dilution with ethanol. Arrows mark the fractions collected as PC hydroperoxides and PE hydroperoxides.

Gas Chromatography and Gas Chromatography/Mass Spectrometry

Fatty acid compositions of phospholipids were determined by gas chromatography after methanolysis using sodium methoxide. The isomeric compositions of hydroxy fatty acids were measured with the aid of gas chromatography/mass spectrometry (GC/MS) after hydrogenation and methanolysis. The procedure for the preparation of the samples for GC/MS analysis is described elsewhere (18,21).

RESULTS AND DISCUSSION

From our preceding study (18), we expected that both PC hydroperoxides and PE hydroperoxides could be separated from unoxidized PC and PE of rat liver by reversed phase liquid chromatography with Lichroprep RP-8. Figure 1 shows the elution patterns of photooxidized rat liver PC and PE. Both chromatograms are very similar to that of photooxidized soybean PC reported previously (18). First and second peak of oxidized phospholipids in Figure 1 appear to be similar to the dihydroperoxides and monohydroperoxides from soybean PC. However, formation of dihydroperoxides involving two fatty acid moieties seems unlikely, because major molecular species of rat liver phospholipids consist of one saturated fatty

acid and one unsaturated fatty acid. Frankel et al. (22,23) have suggested that secondary oxidation proceeds by free radical cyclization and 1,4-addition of singlet oxygen during photosensitized oxidation of methyl linoleate resulting in 5-membered and 6-membered hydroperoxy cyclic peroxides. Furthermore, we previously reported that a second hydroperoxidation could proceed during photosensitized oxidation of arachidonic acid (24). Thus, first peak may consist of oxygenated phospholipids including secondary oxidation products of polyunsaturated fatty acid components. The fractions of eluents corresponding to second peak were collected between 20 and 30 ml from oxidized PC and those between 20 and 26 ml from oxidized PE. Both fractions gave brown spots on a TLC plate when they were sprayed with potassium iodide in ethylcellosolve. This indicated that the eluents contained peroxides and that these compounds were completely separated from unoxidized PC and PE. Thus, these fractions probably included PC hydroperoxides and PE hydroperoxides. The yields of these fractions recovered from the reaction mixture were found to be 19.7% and 16.0% for PC and PE, respectively. Although several reports (13,14,16,17) already have shown that reverse phase HPLC possesses the ability to separate oxidized PC from unoxidized PC, the Lichroprep RP-8 column may be more suitable for the preparative stage because of its larger capacity.

Normal phase HPLC has been used frequently for separating the phospholipid classes (25-27). However, hydroperoxides and hydroxy derivatives usually gave almost the same retention time as that for corresponding unoxidized species (Fig. 2). Thus, normal phase HPLC seems to be inadequate for separating oxidized phospholipids from unoxidized phospholipids. In particular, it was found to be difficult to distinguish the phospholipid hydroperoxides from their hydroxy derivatives with normal phase HPLC. Figure 3 shows the reversed phase TLC of the phospholipid hydroperoxides and their hydroxy derivatives. Each sample was separated to two bands on the plate. This separation may be based on the difference of molecular species of rat liver phospholipids in which two groups, that is, 1-palmitoyl and 1-stearoyl series are known to be principal species (28). Complete separation of hydroxy and hydroperoxy derivatives from their unoxidized phospholipids was observed on the plate. The effectiveness of reversed phase TLC can be predicted from the results of reversed phase HPLC, reported in the literature (13,14,16,17). In addition to reverse phase HPLC, reverse phase TLC may be a convenient and useful method for the detection of phospholipid hydroperoxides.

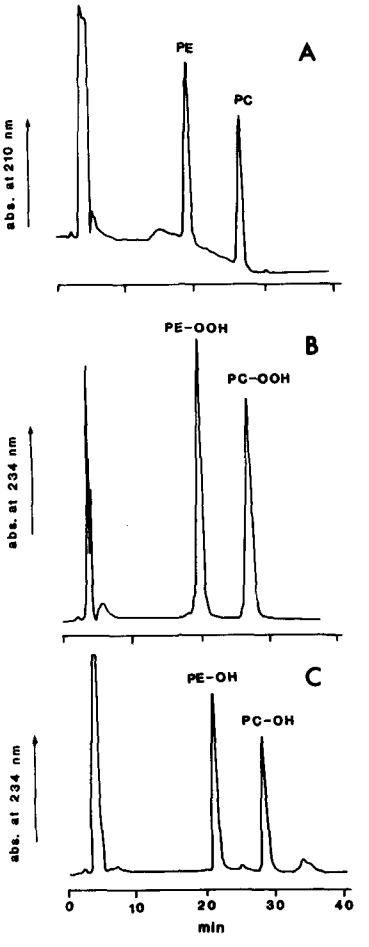

FIG. 2. HPLC elution patterns of PC, PE and their derivatives. A: the mixture of PC and PE; B: the mixture of PC hydroperoxides (PCOOH) and PE hydroperoxides (PEOOH); C: the mixture of hydroxy PC (PCOH) and hydroxy PE (PEOH).

The major fatty acid composition of rat liver PC and PE was found to be palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), arachidonic acid (20:4) and docosahexaenoic acid (22:6), as listed with Table 1. It has been confirmed that $^1O_2$ reacts with olefinic double bonds of unsaturated fatty acid to produce hydroperoxy fatty acid (29). The unsaturated fatty acid components of rat liver phospholipids, that is, 18:1, 18:2, 20:4 and 22:6, seem to be subject to $^1O_2$ oxidation and converted to hydroperoxy fatty acids. Hydroxy fatty acids may be produced by reducing the corresponding hydroperoxy fatty acids in phospholipids.

Structure of the hydroxy fatty acid components was determined by GC/MS analysis after

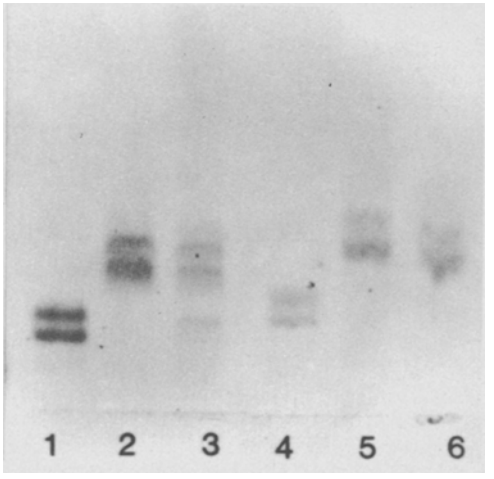

FIG. 3. Reverse phase TLC of PC, PE and their derivatives. Lane 1, PC; lane 2, PC hydroperoxides; lane 3, hydroxy PC; lane 4, PE; lane 5, PE hydroperoxides; lane 6, hydroxy PE.

TABLE 1

Fatty Acid Composition of Rat Liver PC and PE

Fatty acid	As % total by weight[a]	
	PC	PE
16:0	18.2	14.8
18:0	24.9	27.5
18:1	5.3	4.7
18:2	12.4	6.1
18:3	0.3	0.3
20:3	0.6	0.4
20:4	28.7	27.0
20:5	0.5	0.8
22:5	1.0	2.0
22:6	8.2	16.5

[a]The percent of other fatty acids was less than 0.1.

hydrogenation, transesterification and trimethylsilylation, as shown in Figure 4 and Table 2. Peaks 3, 4 and 5 obtained from PC-hydroxy derivatives (Fig. 4-A) were identified as trimethylsilyl (TMS) derivatives of methyl hydroxyoctadecanoate, methyl hydroxyeicosanoate and methyl hydroxydocosanoate, respectively, from their mass spectra (24,30). Principal ions due to the loss of CH_3 from the molecular ion, (M-15), and loss of CH_3 and CH_3OH from molecular ion, (M-47), appeared at m/e (rel. intensity) 371 (7), and 339 (18) in peak 3. A pair of fragment ions due to alpha cleavage of the trimethylsilyloxy group was derived from each isomer of methyl hydroxyoctadecanoate as follows: the 9-isomer, m/e 229 (86), 259 (100); the 10-isomer, m/e 215 (75), 273 (70); the 12-isomer, m/e 187 (76), 301 (53); the 13-

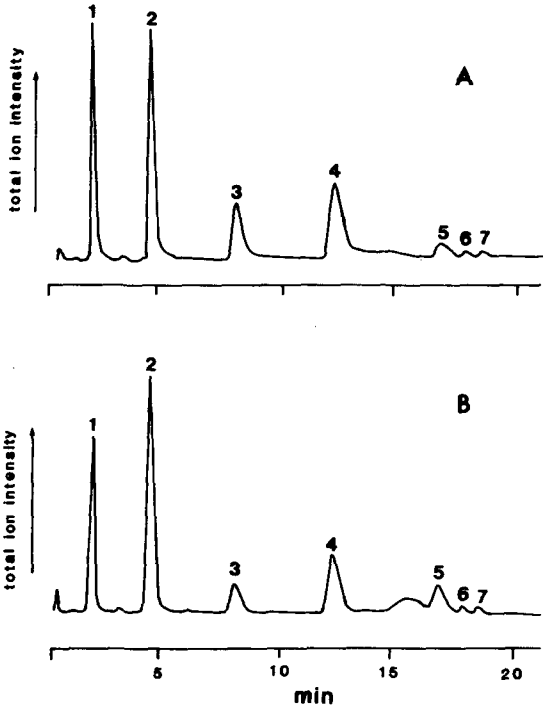

FIG. 4. Detection of TMS derivatives of hydroxy fatty acid methyl ester obtained from hydroxy derivatives of PC (A) and PE (B) after hydrogenation and transmethylation. Peaks identified were as follows: 1, methyl palmitate; 2, methyl stearate; 3, TMS derivatives of methyl hydroxyoctadecanoate; 4, TMS derivatives of methyl hydroxyeicosanoate, and 5, 6, and 7, TMS derivatives of methyl hydroxydocosanoate.

isomer, m/e 173 (20) and 315 (12). The mass spectrum of peak 4 showed (M-15) ion at m/e 399 (15) and (M-47) ion at m/e 367 (26).

Fragment ions due to alpha cleavage of the trimethylsilyloxy group of each regioisomer of hydroxyeicosanoate appeared as follows: the 5-isomer, m/e 203 (39), 313 (14); the 6-isomer, m/e 217 (40), 299 (20); the 8-isomer, m/e 245 (88), 271 (56); the 9-isomer, m/e 259 (74), 257 (57); the 11-isomer, m/e 287 (86), 229 (85); the 12-isomer, m/e 301 (87), 215 (100); the 14-isomer, m/e 329 (37), 187 (54); the 15-isomer, m/e 343 (4), 173 (12). Peak 5 indicated the (M-15) ion at m/e 427 (17) and (M-47) ion at m/e 395 (21). The appearance of the fragment ions due to the alpha cleavage of the trimethylsilyloxy group indicated the following varieties of regioisomers of methyl hydroxydocosanoate: the 7-isomer, m/e 231 (100), 313 (51); the 8-isomer, m/e 245 (83), 299 (19); the 10-isomer, m/e 273 (90), 271 (65); the 11-isomer, m/e 287 (81), 257 (71); the 13-isomer, m/e 315 (71), 229 (89); the 14-isomer, m/e 329 (66), 215 (90); the 16-isomer, m/e 357 (17), 187 (43); the 17-

TABLE 2

Isomeric Compositions of Hydroxy Fatty Acid Components in Hydroxy Phospholipids

Isomer	Relative percent																
	4-OH	5-OH	6-OH	7-OH	8-OH	9-OH	10-OH	11-OH	12-OH	13-OH	14-OH	15-OH	16-OH	17-OH	18-OH	19-OH	20-OH
Hydroxy-PC																	
C-18	–	–	–	–	–	30.5	22.3	–	20.5	26.8	–	–	–	–	–	–	–
C-20	–	4.6	4.8	–	11.5	11.4	–	13.6	14.0	–	16.1	23.9	–	–	–	–	–
C-22	–	–	–	8.7	7.8	9.8	9.8	9.4	–	9.0	9.0	–	11.5	12.2	–	9.0	13.5
Hydroxy-PE																	
C-18	–	–	–	–	–	31.0	23.9	–	20.3	24.8	–	–	–	–	–	–	–
C-20	–	3.3	5.2	–	12.2	12.1	–	14.8	15.1	–	14.9	22.3	–	–	–	–	–
C-22	–	–	–	9.8	9.2	–	10.0	9.6	–	10.1	10.3	–	11.0	10.3	–	8.2	11.7

C-18, methyl hydroxyoctadecanoate; C-20, methyl hydroxyeicosanoate; C-22, methyl hydroxydocosanoate.

isomer, m/e 371 (7), 173 (16). Peaks 6 and 7 were identified as the TMS derivatives of the 19- and 20-regioisomer of methyl hydroxydocosanoate, respectively. A pair of alpha cleavage ions of methyl hydroxydocosanoate appeared at m/e 145 (11), 399 (18) in peak 6 and m/e 131 (100), 413 (27) in peak 7. Peaks 3-7 obtained from the PE hydroxy derivative (Fig. 4-B) had the same fragmentation patterns as those described above. The ratio of the peak area for hydroxyoctadecanoate, hydroxyeicosanoate and hydroxydocosanoate was determined to be 33: 47:20 in the PC hydroxy derivative and 25:50:25 in the PE hydroxy derivative.

The isomeric composition of each hydroxy fatty acid methyl ester was determined by mass chromatographic analysis of the pair of fragment ions (24,29), and the results are shown in Table 2. The isomeric compositions of hydroxyoctadecanoate and hydroxyeicosanoate agreed very closely with those obtained from the oxidation of methyl linoleate (29,31) and methyl arachidonate with $^1O_2$ (24). The finding that the proportion of the 9- and 10-isomers was higher than those expected from methyl linoleate may reflect the contribution of 18:1 to the formation of these isomeric hydroperoxides. On the other hand, 22:6 contains double bonds at 4, 7, 10, 13, 16 and 19 positions and is assumed to produce the 4-, 5-, 7-, 8-, 10-, 11-, 13-, 14-, 16-, 17-, 19- and 20-isomers as hydroxyperoxy docosahexanenoic acid, when the ene type reaction mechanism (32) is applied for the reaction of 22:6 with $^1O_2$. The reason why the 4- and 5-isomers were absent in hydroxydocosanoate is not clear. Hydrogenation might eliminate these hydroperoxide isomers, or a selective attack of $^1O_2$ might occur in the reaction with 22:6 of phospholipids.

From these results, it can be concluded that 18:1, 18:2, 20:4 and 22:6 are all responsible for the formation of hydroperoxides of rat liver phospholipids in $^1O_2$ oxidation. This is supported by the fact that the reactivities toward singlet oxygen among these unsaturated fatty acids are almost proportional to the number of their double bonds (30,33). Another characteristic of $^1O_2$ oxidation is found in the isomeric compositions of hydroperoxy fatty acids. Hydroperoxides specific to $^1O_2$ oxidation are the 10- and 12-isomers from 18:2 (29), the 6- and 14-isomers from 20:4 (24), and presumably the 19-isomer from 22:6. The other isomers listed in Table 2 can be produced by both $^1O_2$ oxidation and free radical oxidation. These differences between the two reactions should be taken into account when this method of preparation is used for experiments of the biological system involving free radical oxidation. Currently we

are investigating lipid peroxidation products produced in vivo by using HPLC, and phospholipid hydroperoxides prepared by the above method are used as standard materials for the determination of the lipid peroxide level.

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COMMUNICATIONS

Stimulation by Lipoxygenase Products of Superoxide Anion Production in FMLP-Treated Neutrophils

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ABSTRACT

Our recent observation that leukotriene B_4 (10^{-9} M) is a potent enhancer of FMLP-initiated neutrophil superoxide anion formation prompted an evaluation of the ability of other lipoxygenase products and related compounds to modulate this response. The results indicate that FMLP-evoked O_2^- may be enhanced by 10^{-8}-10^{-7} M levels of a number of lipids, in addition to LTB_4, including 5-HPETE, 5-HETE, 5,15-DiHPETE and by higher levels of other 15-series lipoxygenase products and arachidonic acid. It is of interest that the relative potency of these agents in potentiating the superoxide response to FMLP approximately parallels their reported ability to induce chemotactic activity in leukocytes. *Lipids* 20:318-321, 1985.

INTRODUCTION

The leukocyte lipoxygenase product 5(S), 12(R)-dihydroxy-eicosa-6,14-*cis*-8, 10-*trans*-tetraenoic acid (leukotriene B_4, LTB_4) is a potent stimulus for neutrophil chemotaxis, chemokinesis and aggregation and enhances the uptake of calcium and D-glucose (1,2). In addition to these primary effects on neutrophil responses, we recently have demonstrated that LTB_4 strongly potentiates (204% at 10^{-9} M) the amount of superoxide anion generated in response to the chemotactic peptide f-met-leu-phe (fMLP) (3). In contrast, Fantone and Kinnes (4) have reported recently that 15-(S)-15-methyl-prostaglandin E_1 and prostaglandin I_2 inhibit fMLP-induced superoxide formation in human neutrophils. Prostaglandin E_1 subsequently has been found to suppress the release of endogenously formed LTB_4 (5).

In the present study we have investigated the effects of a number of other lipoxygenase products and related compounds on the production of superoxide anion in resting and fMLP-stimulated neutrophils. These include a metabolic precursor of LTB_4, 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and 5-hydroxyeicosatetraenoic acid (5-HETE) which is unable to serve as a precursor of LTB_4. 5-HPETE and 5-HETE initiate many of the same neutrophil responses as LTB_4, but are 30-300 times less potent (6). We also tested a group of 15-series lipoxygenase metabolites for their ability to modulate fMLP-induced superoxide formation. Many of these compounds have been identified in extracts from arachidonic acid-stimulated porcine (7) and human (8) leukocytes following incubations with 15-hydroperoxyeicosatetraenoic acid. Subsequent studies have revealed them to be particularly prominent in eosinophils (9). Investigations of biological responses of leukocytes exposed to 15-series products generally have indicated that the compounds are inactive (10,11), although contrary results also have been reported (12). Finally, we have evaluated the specificity of the LTB_4 enhancement of FMLP induced superoxide formation by testing low concentrations of free fatty acids, including arachidonic acid. Arachidonic acid has been reported to act as a primary stimulus for superoxide formation, but concentrations of at least 5μM are required (13,14).

MATERIALS AND METHODS

Ferricytochrome *c* (Type III), fMLP, superoxide dismutase (SOD) and bovine serum albumin (BSA) were purchased from Sigma Chemical Company, St. Louis, Missouri. Hank's balanced salt solution (HBSS) was obtained from the Grand Island Biological Company, Grand Island, New York.

5(R,S)-HETE was synthesized from arachidonic acid via 5-HETE-δ-lactone by methods previously described (15). 5(R,S)-HPETE was produced via autoxidation of arachidonic acid in the presence of vitamin E (16).

15(S)-HETE was formed from 15-HPETE by sodium borohydride reduction. The hydroperoxy derivative was prepared from the sodium salt of arachidonic acid by incubation with soybean lipoxygenase (Type IV, Sigma) in .05 M sodium borate buffer, pH 9.0, for 20 min

at room temperature. The individual HETE and HPETE products were purified by reverse phase HPLC and quantified by UV spectroscopy.

8(S), 15(S)-dihydroperoxy-5,11-*cis*-9,13-*trans*-eicosatetraenoic acid (8,15-DiHPETE) and 5S), 15(S)-dihydroperoxy-eicosatetraenoic acid (5,15-DiHPETE) were prepared from arachidonic acid using soybean lipoxygenase. Arachidonic acid (Supelco Co., Bellefonte, Pennsylvania) was converted to the sodium salt and incubated for one hr in 60 ml of .05N sodium borate buffer, pH 8.7 with soybean lipoxygenase (3.2 mg protein). Products were isolated by reverse phase chromatography monitoring by U.V. detection at 235 nm. Aliquots of each preparation were subjected to reduction by sodium borohydride to form the corresponding dihydroxy derivatives. These compounds were isolated in a similar manner by HPLC.

14,15-LTA_4 was synthesized by methods previously described (17). The 14,15-diHETE (erythro isomer) was prepared by hydrolysis of the leukotriene epoxide and purified by HPLC (7). The major products of hydrolysis were 8,15-diHETE isomers and a yield of about 5% was obtained for the 14,15-diHETE.

12-HETE was obtained by incubating human platelets with arachidonic acid (100 μM) for five min at 37 C. Purification was accomplished by reverse phase HPLC.

Neutrophil Suspensions

Human neutrophils were isolated from heparinized venous blood from healthy adult volunteers as previously described (3).

Neutrophil Superoxide Release

Superoxide (O_2^-) was assayed by measuring the O_2^- dependent (superoxide dismutase-inhibitable) reduction of ferricytochrome *c* (18). All individual experiments were performed in triplicate and repeated as indicated for statistical evaluations. Neutrophils (7.5×10^6 cells/0.5 ml) were incubated for 10 min prior to the addition of a test compound or its solvent control. The cells were then incubated with the lipid for two min and pipetted into cytochrome *c*-solutions (final concentration 0.1 mM) and the cell suspension (1.5×10^6 cells/ml) subsequently exposed to fMLP (10^{-7}M). The cells were then pelleted by centrifugation and the extent of cytochrome *c* reduction in each supernatant was determined by the change of absorbance at 550 nm (Gilford 250 recording spectrophotometer) in reference to blanks to which superoxide dismutase was added prior to the oxidative stimulus. Superoxide concentrations were calculated using the value of 15.5 $(nM \cdot cm)^{-1}$ for the difference in extinction coefficients of the reduced and oxidized forms of cytochrome *c*.

Addition of Test Lipids

Test lipids were evaporated to dryness and redissolved by sonication in 50 mg/ml BSA. A 250 microliter aliquot of this solution was added to 500 microliters of 7.5×10^6 cells and incubations performed as described above.

RESULTS

The low resting level of neutrophil superoxide anion production was not enhanced by pre-exposure of the cells to 5-HETE or 5-HPETE, whereas fMLP-treated neutrophils produced significant levels of O_2^- (Table 1). Despite the lack of effect on resting neutrophils, pre-exposure of neutrophils to 5-HETE or 5-HPETE (10^{-8}-10^{-6}M) resulted in a greatly augmented level of superoxide anion production in response to fMLP (Table 2). A variety of 15-series lipoxygenase-derived compounds and selected free fatty acids were also tested as potential modulators of fMLP-induced superoxide formation. All compounds were ineffective at inducing O_2^- production in the absence of fMLP (data not shown). 15-HETE and most of the other 15-series metabolites slightly enhanced O_2^- release at 10^{-6}M concentrations (Table 2). The dihydroperoxy derivative 5, 15-diHPETE was somewhat more potent as an enhancer at O_2^- formation in fMLP-treated cells, exhibiting a potency comparable to 5-HETE. Arachidonic acid and other free fatty acids also slightly enhanced the superoxide response of fMLP-treated neutrophils at a concentration of 10^{-6}M. The previously reported

TABLE 1

Effects on Superoxide Anion (O_2^-) Release of Exposure of Neutrophils to 5-HETE, 5-HPETE or fMLP

Agent[a]	O_2^- (nmoles/1.5 × 10^6 cells)
None	3.2 ± 0.84 (4)[b]
5-HETE (10^{-6}M)	2.5 ± 0.58 (4)
5-HPETE (10^{-6}M)	3.6 ± 0.34 (4)
FMLP (10^{-7}M)	12.0 ± 1.5 (12)[c]

[a]Test for lipids or their solvent control were preincubated with human neutrophils (7.5×10^6 cells/0.75 ml) for 2 min prior to dilution of the cells into the superoxide assay medium.

[b]Mean ± S.E.M. The number of individual experiments is given in parentheses.

[c]Values significantly different from control ($P < .001$).

TABLE 2

Effects of Selected Lipoxygenase Products and Related Lipids on O_2^- Production by Neutrophils Following Treatment with fMLP

	FMLP-induced O_2^- (% of control)[a]			
	Concentration (M)			
Agent[b]	10^{-6}	10^{-7}	10^{-8}	10^{-9}
5-HETE	175 ± 19.0 (3)[c]	152 ± 10.0 (8)[c]	121 ± 7.4 (7)[c]	101 ± 7.4 (7)
5-HPETE	176 ± 40.0 (4)[c]	185 ± 25.1 (6)	135 ± 12.3 (4)[c]	106 ± 4.5 (4)
8,15-DiHETE	132 ± 13.5 (5)[c]	115 ± 7.9 (5)	114,108	ND[d]
5,15-DiHETE	130 ± 14 (4)[c]	105 ± 0.5 (4)	106,118	99,104
8,15-DiHPETE	139 ± 28.7 (4)	139 ± 28.7 (4)	ND	ND
5,15-DiHPETE	189 ± 21.3 (4)[c]	141 ± 9.7 (4)[c]	116 ± 4.4 (3)	102 ± 0.8 (3)
14,15-DiHETE	134 ± 5.1 (3)[c]	111 ± 7.1 (3)	106 ± 2.2 (3)	103,110
15-HETE	140 ± 13.2 (4)[c]	106 ± 2.3 (4)	103	ND
Arachidonic acid	144 ± 7.4 (4)[c]	105,102	100,100 (2)	100.5, 100 (2)
20:3(n-9)	132,144	112,100	ND	ND
Linoleic acid	110,104	100,101	ND	ND
12-HETE	172,159	119,110	100,99	99,100

[a]Mean ± S.E.M. of percent of control (incubations with BSA). The number of experiments is given in parentheses. Individual data points are given for compounds in which less than 3 assays were performed.

[b]Test lipids were preincubated for 2 min with human neutrophils (7.5×10^6 cells/0.75 ml) prior to dilution of the cells into the superoxide assay medium, fMLP stimulation and superoxide anion determination as described in the text.

[c]Values significantly different from paired control ($P < .05$).

[d]ND signifies concentrations of a given agent not tested.

inhibition of superoxide production by PGE_1 (4) in neutrophils treated with fMLP was observed, with a reduction to 57% of control seen at 1 μM PGE_1.

DISCUSSION

Leukotriene B_4 is a potent neutrophil chemoattractant but is a weak inducer of O_2^- production (6) requiring concentrations 1000-fold greater than those necessary to induce neutrophil chemotactic responses. Nevertheless, very low concentrations (10^{-9}M) effectively enhance the substantial degree of superoxide production from cells stimulated with fMLP (3). The present study has shown that a number of other lipoxygenase products and related lipids are also able to enhance fMLP-evoked superoxide production despite being ineffective as a primary stimulus. It is of interest to note that the relative potency of many of these compounds in this regard roughly parallels their potency as neutrophil chemoattractants. Thus, 5-HPETE and 5-HETE, which require approximately 300-fold greater concentrations to evoke chemotactic activity (6), were two orders of magnitude less effective as enhancers of fMLP-initiated superoxide formation. Similarly, the dihydroxy leukotriene metabolites related to 14,15-LTA_4 require micro molar levels to evoke a chemotactic response (10) and to enhance FMLP-induced superoxide production.

The potentiation of fMLP-evoked superoxide anion formation by the lipids investigated in this study were thus of a lower potency than LTB_4. Nevertheless, some of these compounds may achieve considerably higher concentrations than LTB_4 and may indeed be of physiological importance. In particular, synovial fluids of patients with rheumatoid arthritis contain elevated levels of 5-HETE in the micromolar range (19), a concentration which augments fMLP-initiated superoxide formation. The hydroperoxy derivative, 5-HPETE, was also a potent enhancer of fMLP-induced superoxide production although the possibility exists that the observed activity was mediated by its potent metabolite, LTB_4. In view of the substantial activity of 5,15-DiHPETE, one might surmise that 5-HPETE could exhibit an enhancing effect independent of LTB_4 synthesis.

The ability of lipoxygenase products and related lipids to enhance the formation of superoxide anion by leukocytes in response to fMLP suggests that these compounds may act primarily as modulators of biological activities induced by other agents rather than as direct

stimuli. In this regard, O'Flaherty, et al. recently reported that 5-HETE and 5-HPETE markedly potentiate the degranulation response of neutrophils to platelet activating factor (PAF) or to LTB_4, although 5-HETE lacked intrinsic degranulating activity and 5-HPETE only weakly evoked degranulation (20,21). The mechanism by which these and other lipids enhance fMLP-evoked O_2^- appears not to involve a change in fMLP receptor binding (3). Further studies regarding lipid modifiers and neutrophil function are in progress.

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Relationship between Platelet Cyclooxygenase Pathway and Plasma Malondialdehyde-Like Material

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ABSTRACT

Thrombin-induced platelet malondialdehyde (MDA) formation and plasma malondialdehyde-like material (MDA-LM) were evaluated in 12 healthy subjects before and after 1 and 7 days from aspirin (1 g) ingestion. 24 hr after aspirin administration, platelet MDA was almost abolished while MDA-LM showed a 23% decrease. Platelet MDA and plasma MDA-LM returned to baseline values 7 days after aspirin ingestion. These data suggest that platelet cyclooxygenase pathway affects only in part plasma MDA-LM. The evaluation of plasma MDA-LM before and after aspirin could be useful for evaluating in vivo platelet cyclooxygenase activation.
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INTRODUCTION

Several forms of atherosclerotic disease show high values of plasma malondialdehyde-like material (MDA-LM); these abnormalities have been checked in cerebrovascular and coronary heart disease (1-4). The interpretation of these results is not unique; some authors considered this MDA-LM behavior as expression of platelet cyclooxygenase (PC) hyperfunction (2), while others suggested that plasma MDA-LM does not necessarily reflect PC activability (1,2). However, so far these different interpretations could be due to the different method used for evaluating plasma MDA-LM (5). In fact, performing plasma MDA-LM in the supernatant only or in both supernatant and protein precipitate could imply the investigation of several metabolic pathways leading to the malondialdehyde (MDA) formation.

Recently we investigated plasma MDA-LM in the supernatant only, showing that this is a reliable index of blood lipid peroxides (5). Because the origin of plasma MDA is not fully understood, we attempted to evaluate whether a relationship between PC activability and plasma MDA-LM does exist. This study shows that plasma MDA-LM only in part reflects PC activation.

MATERIALS AND METHODS

In Vivo Study

The investigation was carried out on 12 healthy volunteers (8 males, 4 females, age 20-39 years) not taking any drugs for at least one month. Blood samples were withdrawn, without stasis, from each subject after fasting 12 hr, mixing 9 parts of blood with 1 part of 0.13 mol Na-citrate. Platelet rich plasma was obtained after centrifugation at 150 × g for 10 min. Platelet MDA formation was studied after thrombin (0.5 U/ml) induction, following Smith's method (6). Plasma MDA-LM was evaluated on platelet poor plasma (PPP) as previously described (5), and expressed as μM. The day-to-day coefficient of variation of MDA-LM was 10%. The MDA-recovery determined by adding different quantities of MDA (Malondialdehyde Tetramethyl acetal, Eastman Kodak Co., Rochester, New York) to human plasma was 88% (5). After platelet MDA and plasma MDA-LM evaluation, all subjects were given 1 g of aspirin. Controls of platelet MDA and plasma MDA-LM were made after 24 hr and 7 days from the aspirin administration.

In Vitro Study

Since during centrifugation PC can be activated by mechanical stimulation (7), we evaluated whether this could affect plasma MDA-LM values. Citrated blood samples from 4 healthy subjects were incubated with aspirin (1 mM) or with physiological salt solution, as control, and then centrifuged at 2000 × g for 15 min for obtaining PPP. Plasma MDA-LM was determined in both samples. Mean, SD, Student's "t" test for paired data were calculated.

RESULTS

In Vivo Study

Platelet MDA formation was almost abolished in all subjects 24 hr after aspirin ingestion (Fig. 1). In only 3 subjects it was possible to detect a very small amount of MDA. The platelet MDA formation was fully recovered in the

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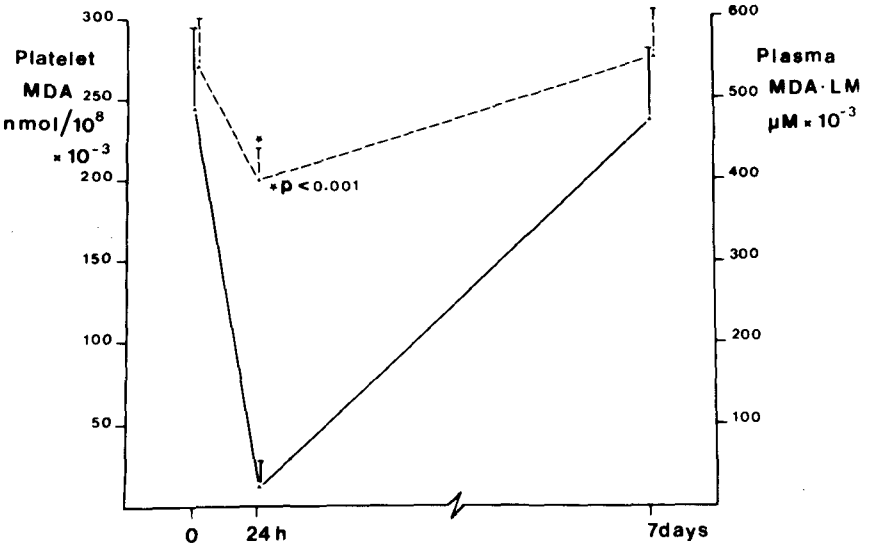

FIG. 1. Platelet MDA values (nmol/10^8 platelets; mean ± SD) before (0.243 ± 0.048) and after 24 hr (0.011 ± 0.015) and 7 days (0.237 ± 0.046) after aspirin ingestion (———). Plasma MDA-LM values (μM; mean ± SD) before (0.539 ± 0.080) and after 24 hr (0.417 ± 0.060) and 7 days (0.541 ± 0.078) after aspirin ingestion (– – – – – –).

control performed 7 days after aspirin ingestion (Fig. 1). A significant decrease of plasma MDA-LM was observed 24 hr after aspirin ingestion (Fig. 1). The lowering of plasma MDA-LM was seen in all subjects and was in mean 23% (range 16-34). The amount of MDA-LM decrease obtained after aspirin ingestion and probably attributable to the in vivo activity of PC was 0.122 ± 0.04 μM (Mean ± SD) (range 0.068-0.203 μM). Seven days after aspirin administration, plasma MDA-LM reached baseline values (Fig. 1).

In Vitro Study

Similar plasma MDA-LM values were seen in citrated blood samples added with aspirin (0.525 ± 0.068 μM; Mean ± SD) or with physiological salt solution (0.534 ± 0.071 μM; Mean ± SD).

DISCUSSION

Platelet MDA is a metabolite of cyclooxygenase pathway (8) and it is retained an index of PC activability (9-11). Aspirin that inhibits PC in a dose dependent manner (12) simultaneously prevents the formation of cyclooxygenase products, such as thromboxane A_2 and MDA (11).

Aspirin administration in man reduces or completely abolishes platelet MDA formation, according to the dose of aspirin ingested (12). The platelet MDA lowering that is due to the aspirin acetylation of PC (13) is time dependent; 7 days after a single ingestion of a large dose of aspirin, i.e. 1 g, the platelet MDA formation is almost recovered (12). Our investigation agrees with these observations since platelet MDA was almost abolished 24 hr after aspirin ingestion and returned to baseline values after 7 days. During the phase of platelet MDA inhibition we observed a significant lowering of plasma MDA-LM; this reduction, however, affected only in part the baseline plasma MDA-LM values, because a decrease of only 23% was detected. Simultaneous to the return of platelet MDA formation to the baseline values, plasma MDA-LM levels were fully recovered. These data suggest that plasma MDA-LM reflect in part the in vivo activation of PC pathway. The remaining plasma MDA-LM probably has another origin, even though we cannot exclude that other platelet pathways, as lipooxygenase pathway, could affect the plasma MDA-LM.

However, the evaluation of plasma MDA-LM before and after aspirin ingestion could represent a simple method for the in vivo study of PC pathway. So far PC pathway has been studied after in vitro platelet stimulation (14, 15), while in our experimental procedure plasma MDA-LM levels are not dependent on in vitro platelet stimulation. This is further sug-

gested by the in vitro study; in fact, during mechanical stimulation as during the centrifugation, platelets can be activated (7); this stimulation probably is not enough to induce changes in plasma MDA-LM. In fact, citrated blood samples with aspirin added had MDA-LM values similar to controls, suggesting that during centrifugation platelet does not release MDA in such a manner that it affects plasma MDA-LM.

Therefore, the evaluation of plasma MDA-LM in the supernatant can give information on the concentration of blood lipid peroxides not crosslinked with proteins (5); the amount of MDA-LM that is suppressed by aspirin ingestion could account for the in vivo activability of PC. This differentiation could be useful for evaluating whether the increase of plasma MDA-LM in several atherosclerotic conditions is attributable to the PC activability.

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Activities of Liver Mixed Function Oxidase System in Rats Fed *trans* Fat

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ABSTRACT

Rats were fed diets containing either camellia oil or partially hydrogenated corn oil as a source of *cis* or *trans* octadecenoate, respectively, in the presence of adequate linoleic acid. After 35 days of feeding the diets, activities of several hepatic drug metabolizing enzymes as well as the content of hepatic microsomal cytochrome P-450 were determined. Geometrical difference in the dietary fat did not affect the amount of microsomal protein nor the content of cytochrome P-450. Also, activities of NADPH cytochrome C reductase, aminopyrine N-demethylase and biphenyl hydroxylase were approximately the same between two groups of rats. Aniline hydroxylase was slightly elevated in the rats fed *trans* fat. It was concluded that the difference in the geometry of dietary fatty acids had little effect in modulating the hepatic mixed function oxidase system.
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INTRODUCTION

Epidemiological (1,2) as well as experimental studies in animals (3-5) have shown that there is a positive correlation between incidence of several types of cancer and quality and quantity of dietary fat. Many carcinogenic substances acquire their potency to induce tumors only after being metabolized by the mixed function oxidase system in microsomes (6). Types and amounts of fat ingested not only affect the activities of the enzyme system (7-10), but also alter the extent of tumor development induced by chemical carcinogens in laboratory animals (3-5).

The hydrogenation products of edible oils usually contain variable amounts of *trans* fatty acid mainly as *trans* octadecenoate (11). It is well established that the degree of unsaturation of dietary fat alters the activities of mixed function oxidases (7-10) and incidence of cancer (3-5). Although a few animal experiments with chemical carcinogens showed no specific stimulatory effects of *trans* fat as compared to *cis* fat (4,5), an epidemiological survey (though not conclusive) claimed positive correlation between the amount of *trans* fatty acids consumed and cancer causation (12). In case *trans* fat modulates the cancer causation, it also may alter the drug metabolizing system. In this context, the effect of dietary *trans* fat on the activities of the hepatic mixed function oxidase system was examined in rats in the present study.

MATERIALS AND METHODS

Animals and Diets

Male Sprague-Dawley rats (Seiwa Experimental Animals, Ltd., Fukuoka) initially weighing about 100 g were divided into two groups and given experimental diets and water freely. The composition of the experimental diets was in weight percent: fat, 10; casein, 20; mineral mixture, 4; vitamin mixture, 1; cellulose powder, 2; choline chloride, 0.15, and sucrose, to 100. Mineral and vitamin mixtures were according to Harper (13) and purchased from Oriental Yeast Co., Tokyo. Dietary fats used were camellia oil and partially hydrogenated corn oil for the sources of *cis* and *trans* octadecenoate, respectively. As the amount of linoleic acid profoundly affects the activities of mixed function oxidases (7-10), the linoleic acid content was adjusted to be the same by supplementing with safflower oil. The fatty acid composition of the diets is shown in Table 1.

Enzyme Assays

After 35 days on the experimental diets, blood was withdrawn under light ether anesthesia at around 10 a.m. from the abdominal aorta. A cannula was inserted into the portal vein and the liver was perfused in situ with 0.9% NaCl to

TABLE 1

Fatty Acid Composition of Diets

	(weight %)	
	cis fat	*trans* fat
16:0	8.3	10.6
18:0	2.1	7.7
t-18:1	trace	24.9
c-18:1	69.1	35.9
cc-18:2	19.9	19.9

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remove the remaining blood. A portion of liver was homogenized in 3 volumes of 10 mM phosphate buffer (pH 7.4) containing 1.15% KCl. The homogenate was centrifuged at 12,000 × g for 10 min. The supernatant obtained was used for the determination of the activities of aminopyrine N-demethylase (14), aniline hydroxylase (15) and biphenyl hydroxylase (16). Another portion of liver was homogenized in 9 volumes of 10 mM phosphate buffer (pH 7.4) containing 1.15% KCl and 10 mM EDTA and centrifuged at 12,000 × g for 20 min. The supernatant was centrifuged at 105,000 × g for 60 min to obtain microsomes. The microsomes were suspended in 50 mM phosphate buffer (pH 7.2) containing 1 mM EDTA for the determination of the activities of NADPH cytochrome C reductase (17) and the content of cytochrome P-450 (18).

Materials

NADP, NADPH, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were from Oriental Yeast Co., Tokyo. Cytochrome C (Type III) was from Sigma Chemical Co., St. Louis, Missouri.

RESULTS AND DISCUSSION

There were no differences in the growth and food intake between the two groups of rats. Liver weight and the content of hepatic microsomal protein and cytochrome P-450 also were comparable (Table 2). Activities of several drug metabolizing enzymes and NADPH cytochrome C reductase are summarized in Table 3. As this table shows, the geometry of fat was relatively irrelevant to the mixed function oxidase system. Among the various enzymes tested, none of the enzymes except aniline hydroxylase, in which the activity was slightly elevated on feeding *trans* fat, was altered by the geometrical difference in the dietary fat.

Dietary fats have been reported to enhance the mixed function oxidases in the liver and increase the content of hepatic cytochrome P-450 (7-10). Also, fat feeding increases the rate of the conversion of the several types of carcinogen to its active form in the liver, intestine and kidney of rats (19-21). These modulations may be brought about through the changes in the nature of microsomal membranes (9). Polyunsaturated fats such as corn oil and safflower oil are particularly effective at enhancing drug metabolizing enzymes compared to saturated fats such as lard or tallow (7-10). These responses correlate well with the higher incidences of several types of cancer in experimental animals fed polyunsaturated fat (3-5). Although one study reported suppression of the mixed function oxidase system by intraperitoneally injected elaidic and linoleic acids in

TABLE 2

Effect of *trans* Fat on Liver Weight and Amounts of Hepatic Microsomal Protein and Cytochrome P-450[a]

	Liver weight (g/100 g body weight)	Microsomal protein (mg/g liver)	Cytochrome P-450 (nmol/mg protein)
cis fat (7)[b]	4.89 ± 0.19	25.1 ± 1.5	1.30 ± 0.11
trans fat (8)	5.15 ± 0.19	23.5 ± 1.3	1.04 ± 0.07

[a]Values represent means ± S.E.
[b]Number of rats in parentheses.

TABLE 3

Effect of *trans* Fat on the Activities of Drug Metabolizing Enzymes[a]

	(nmol/min/g liver)			(μmol/min/mg protein)
	Aminopyrine N-demethylase	Aniline hydroxylase	Biphenyl hydroxylase	NADPH cytochrome C reductase
cis fat (7)[b]	0.724 ± 0.054	354 ± 33	50.8 ± 6.9	38.3 ± 2.3
trans fat (8)	0.645 ± 0.040	483 ± 27[c]	52.2 ± 4.0	36.8 ± 1.2

[a]Values represent means ± S.E.
[b]Number of rats in parentheses.
[c]Significantly different from the value in rats fed *cis* fat at $p < 0.05$.

rats fed a fat-free diet (22), no information is available on the effect of the geometry of dietary fat. As *trans* octadecenoate compared to the *cis* counterpart is incorporated more readily into hepatic phospholipids (23), feeding of *trans* fat may modulate the function of biological membranes. Under the dietary conditions essentially similar to the present study, we found that as much as 10% of fatty acids in microsomal phospholipids were replaced by *trans* octadecenoates (24). Hsu and Kummerow (25) reported altered mitochondrial enzyme functions in rats fed partially hydrogenated vegetable fat. Decker and Mertz (26) found altered swelling properties of hepatic mitochondria and hemolytic rates of erythrocytes from rats fed *trans* fat. However, the present study showed *trans* fat did not affect the function of microsomes, at least with respect to the drug metabolizing system. In this context, we found that *trans* octadecenoate was incorporated into hepatic microsomal phospholipids at the expense of saturated fatty acids rather than of *cis* octadecenoate insofar as the supply of linoleic acid was adequate (24). Thus, fluidity of the membrane was considered to be unaltered. In the same report, we reported enhanced activities of microsomal 3-hydroxy-3-methylglutaryl CoA reductase and cholesterol 7α-hydroxylase in the liver of rats fed *trans* fat. However, these alterations were ascribable to the enhanced fecal steroid excretion rather than to the changes in the nature of microsomal membrane in rats fed *trans* fat (24,27).

Brown (4) reported that dietary *trans* fat did not affect the dimethylhydrazine induced and spontaneous liver tumor incidences in mice compared to *cis* fat. Also, Selenskas et al. (5) obtained similar results in the incidences of mammary cancer induced by dimethylbenz(a)-anthracene in rats. The present observation that *trans* fat did not affect the hepatic drug metabolizing system favors these observations.

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Increased Arachidonate Incorporation in Perfused Heart Phospholipids from Vitamin E-Deficient Rats

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ABSTRACT

When perfused with exogenous arachidonic acid (AA), the rat heart incorporates this eicosanoid precursor into the phospholipids. The incorporation of AA into phosphatidylcholine was 6-fold higher than the incorporation of AA into phosphatidylethanolamine. When vitamin E-deficient rat hearts were perfused with AA, there was a marked increase in incorporation of AA into both phosphatidylcholine and phosphatidylethanolamine compared to rats fed with a vitamin E supplemented diet. The result of this study suggests that vitamin E has a regulatory role in phospholipid biosynthesis in the mammalian heart.
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INTRODUCTION

The phospholipids play an important role in membrane structure and function, and the fatty acid composition of membrane phospholipids is continuously modified by intrinsic and extrinsic factors such as hormones and diet. Recent experimental evidence from different laboratories indicates that the metabolic turnover of arachidonic acid, the major precursor of the eicosanoids, can be influenced by the vitamin E status of the animal (1-5). Vitamin E appears to regulate eicosanoid production not only at the cyclooxygenase and lipoxygenase steps but also at the release of arachidonic acid via phospholipase A_2 (6,7). However, the role of vitamin E in the incorporation of arachidonic acid into the myocardium lipids is not known. This report presents evidence that vitamin E may have a regulatory role in the incorporation of arachidonate into the cardiac phospholipids.

MATERIALS AND METHODS

Chemicals

All chemicals were of reagent grade and organic solvents were redistilled. [1-^{14}C]-Arachidonic acid (56 mCi/mmole) was obtained from New England Nuclear Canada (Lachine, Quebec). Diet ingredients were from ICN Nutritional Biochemicals (Cleveland, Ohio). Unlabeled arachidonate and snake venom *(Naja naja)* phospholipase A_2 were from Sigma (St. Louis, Missouri) and the arachidonate was purified by silicic acid column chromatography before use.

Animals and Diets

One-month-old male Sprague-Dawley rats were fed for 3 mo on a semi-purified diet containing in weight %: vitamin-free casein, 20; dextrose, 50; starch, 10.2; cellulose, 5; stripped corn oil, 10; AIN salt mix, 3.5 (8); vitamin mix (tocopherol free), 1.0 (5); and DL-methionine, 0.3. Controls were fed the same diet enriched with 100 ppm of [dl]-α-tocopherol acetate. Animals were housed individually and were given tap drinking water and diet at all times. Vitamin E deficiency was verified by plasma tocopherol concentration measured by HPLC as described previously (9). Plasma tocopherol values were 1.30±0.4 and 0.20±0.01 mg/dl for vitamin E sufficient and deficient rats, respectively.

Heart Perfusion

The rat was anesthetized with ether, incised and blood was collected by a lower abdominal aorta puncture. The heart was rapidly excised and rinsed twice with ice-cold Krebs-Henseleit buffer (10) previously saturated with 95% O_2:5% CO_2. After removal of connective tissues and fat, the aorta was cannulated and immediately perfused in the Langendorff mode (11) with oxygenated Krebs-Henseleit buffer for 12 min for stabilization. At this time, 15 ml of arachidonate enriched Krebs-Henseleit buffer was introduced (contained 10 mg bovine serum albumin, 250 μg unlabeled and 1 μCi labeled arachidonate). The effluenct was recycled for 30 min with constant oxygenation. The perfusate was maintained at 37±1 C with a flow rate of 3-3.2 ml per min. The viability of the perfused heart was assessed as previously described (12).

Quantitation of Heart Lipids

The hearts were weighed immediately after

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perfusion and were either frozen in liquid nitrogen or homogenized in a tissumizer (Tekmar, SDT-1810) with 15 ml of chloroform:methanol (1:2, v/v). Total heart lipids were extracted twice using the Bligh and Dyer method (13). The pooled $CHCl_3$ fractions were divided in 3 ml aliquots and stored at -20 C until analyzed (less than 1 week). Phospholipids were separated by thin layer chromatography (TLC, silica gel G, 250 μm) using a solvent system of chloroform:methanol:water:acetic acid (85:15:10:3.5, v/v/v/v). Diacylglycerol and free fatty acids were separated by a solvent system of ether:diethylether:acetic acid (85:15:1, v/v/v). Different lipid classes were identified by comigration of unlabeled lipid standards under iodine exposure. Radioactivity of different bands was determined by liquid scintillation counting. The data were analyzed using the Student's t test.

Positioning of Incorporated Fatty Acid

In order to determine the positioning of the incorporated arachidonate in the phospholipids, phosphatidylcholine (PC) was separated from total heart lipid by TLC and eluted with chloroform:methanol (1:1, v/v). The recovered PC was subjected to hydrolysis by snake venom phospholipase A_2 *(Naja naja)*, and the hydrolyzed free arachidonate was separated by TLC and quantified by scintillation counting. To determine whether the labeled arachidonic acid had been metabolized prior to or after incorporation, samples of heart lipids were transmethylated with methanol-HCl and the methyl esters were determined by a gas-chromatogram equipped with a radiodetector.

RESULTS

The heart weights of the vitamin E deficient and vitamin E sufficient rats as well as the incorporation of arachidonic acid (AA) into the cardiac neutral lipids of these two groups of animals are presented in Table 1. Although linearity of AA uptake was not maintained after 5 min of perfusion, a valid comparison of incorporated labeled material between these two animal groups is still feasible because the heart weights of the vitamin E-deficient and sufficient rats were not significantly different. Apart from an increase in total fatty acid uptake, the vitamin-E deficient heart also showed a significantly higher incorporation of perfused arachidonate into diacylglycerol and triacylglycerol when compared to the vitamin E-sufficient animals. However, the amount of free labeled arachidonate remained relatively unchanged. In the cardiac phospholipid fractions, there was a marked preferential incorporation (over 6 fold) of arachidonic acid into phosphatidylcholine over phosphatidylethanolamine (Fig. 1). Vitamin E deficiency elicited a significantly higher incorporation of perfused arachidonate into both phosphatidylcholine (PC) and phosphatidylethanolamine (PE). However, the ratio of PC to PE remained unchanged within each treatment group (6.5 for the vitamin E-deficient and 7.3 for the vitamin-E supplemented rats).

In order to determine the positioning of the incorporated fatty acid in these phospholipids, selected cardiac PC was subjected to hydrolysis by snake venom phospholipase A_2. Complete hydrolysis of labeled fatty acid from PC indicated that arachidonate was esterified exclusively at the sn-2 position. To investigate

TABLE 1

Heart Weight and the Incorporation of [1-^{14}C] Arachidonate into Neutral Lipids of the Perfused Rat Heart[a]

	Vitamin E-sufficient	Vitamin E-deficient
	grams	
Heart weight	1.66±0.1	1.53±0.30
	(dpm × 10^3/gm heart)	
Total fatty acid uptake	700±85	1000±100*
Free fatty acid	160±28	195± 43
Diacylglycerol	25± 3	40± 4**
Triglyceride	216±61	407± 45**

[a]Values are mean ± SEM of 3 animals per group. *Significantly different $P<0.05$ and **$P<0.025$.

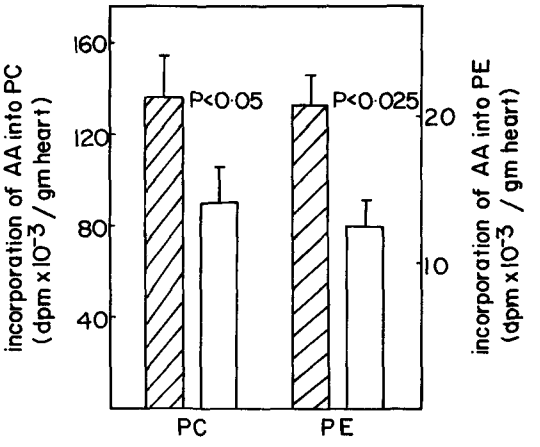

FIG. 1. Incorporation of arachidonate in phospholipids of the isolated perfused hearts from rats fed vitamin E-deficient (▨) and supplemented (□) diets. Data represent mean ± SEM of 3 animals per group. PC (phosphatidylcholine) and PE (phosphatidylethanolamine). Details of heart perfusion are in the text.

whether the labeled arachidonic acid had been metabolized prior to or after the incorporation, samples of heart lipids were transmethylated. The radioactive profile of the perfused heart lipid consistently showed one single radioactive peak with the same retention time of methylated [^{14}C]-arachidonate, indicating that there was undetectable transformation of incorporated arachidonate.

DISCUSSION

PC and PE are the major phospholipids in the mammalian heart. Beyond their role as building blocks for the cardiac membrane, these phospholipids are also potential sources of arachidonic acid, the substrate precursor of the biologically very active eicosanoids. In the present study, increased incorporation of arachidonate into both cardiac PC and PE was observed in the vitamin E-deficient rats. Such an increase cannot be attributed to heart size because there was no difference between the heart weights of these two groups. It can be argued that the increase of labeling material in phospholipids during vitamin E-deficiency was not caused by a higher rate of incorporation but from a diminished intracellular free AA pool found in the heart of vitamin E-deficient rats. However, this situation is most unlikely because the concentration of free AA in mammalian heart is very low (10-20 nmol/gm ref. 16), and hence would not be able to account for the observed increase in the labeling of cardiac phospholipids even if there were a substantial increase of free AA pool in the vitamin E-deficient animals.

Although the effect of vitamin E on the catabolism of phospholipids has been demonstrated recently (6), this is the first report on the regulatory role of vitamin E in phospholipid biosynthesis in the mammalian heart. At present, the exact mechanism for the increased incorporation of arachidonate into cardiac phospholipids due to vitamin E-deficiency is not known. However, the increase in labeling of the phospholipids may result from the greater influx of arachidonate, as shown in Table 1. The resultant increase in the labeling of diacylglycerol inevitably would cause increases in the labeling of PC and PE through de novo synthesis (12,14).

In addition, these cardiac phospholipids have been shown to go through an extensive deacylation-reacylation process (15), and arachidonyl CoA is the preferred substrate for the reacylation reaction (16). Hence, it is conceivable that the deacylation-reacylation process may also play a significant role in the increased incorporation of arachidonate into PC and PE during vitamin E deficiency.

The results of this study confirm previously published work in which a higher proportion of arachidonic acid was found in tissue phospholipids during vitamin E-deficiency (17,18). Moreover, it extends the role of vitamin E in the arachidonic acid cascade beyond the already demonstrated cyclooxygenase and lipoxygenase pathways (1-5).

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Glycosphingolipids of Fetal and Adult Sheep Colonic Mucosa

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ABSTRACT

The ganglioside and neutral glycosphingolipid composition of fetal and adult sheep colonic mucosa were characterized and compared. Mono- and tetrahexosylceramide were the major neutral glycolipids of both fetal and adult colons. Adult, but not fetal, mucosa also possessed di- and trihexosylceramide. Similarly, GD_{1a}, GM_3 and GM_2 were found to be the principal gangliosides in fetal and adult tissue. Adult colonic mucosa possessed significant amounts of GT_{1a} not present in fetal tissue.

Analysis of the hydroxy and nonhydroxy fatty acids as well as of the long chain bases of the major glycosphingolipids revealed differences between these lipophilic components of glycolipids in fetal and adult colonic mucosa. The present results, therefore, indicate that both quantitative and qualitative differences in glycosphingolipid composition exist between fetal and adult sheep colonic mucosa.
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INTRODUCTION

Glycosphingolipids may be involved in a number of important phenomena such as cellular adhesion, transmembrane transport, cell growth, contact inhibition, binding by cells of hormones, enzymes and toxins, and malignant transformation (1,2). In recent years a number of studies have demonstrated alterations in the glycolipid composition of the small intestinal mucosa during normal differentiation (3-5) and development (6-8), suggesting a possible role for glycosphingolipids in these processes.

While the colon also is recognized as an important organ with respect to both normal physiological and pathological processes, to date little is known concerning the possible role of glycosphingolipids in the normal development of this organ.

The present studies, therefore, were undertaken to determine whether differences exist in the glycosphingolipid composition of fetal and adult sheep colonic mucosa. The results demonstrate that quantitative and qualitative differences in the glycolipid composition of fetal and adult sheep colonic mucosa exist and serve as the basis for the present report.

MATERIALS AND METHODS

Materials

Mixtures of nonhydroxy and hydroxy fatty acid standards, EGSS-X 10% on Gas Chrom P and Power-Sil-Prep were obtained from Alltech/Applied Sciences Lab Inc., Arlington Heights, Illinois. Sphingosine standards and OV-1 3% on Anakron ABS were purchased from Foxboro/Analabs, Inc., North Haven, Connecticut. Phytosphingosine was obtained from Calbiochem, San Diego, California. Florisil (60-100 mesh) and Fluorescamine (Fluram-Roche) were obtained from Fisher Scientific, Fairlawn, New Jersey.

The ganglioside standards GM_1, GD_{1a}, GT_{1a} and GT_{1b} were purchased from Supelco Inc., Bellefonte, Pennsylvania. GM_2 was a gift from Dr. G. Dawson isolated from Tay-Sachs brain. GM_3 was isolated and purified by the method of Glickman and Bouhours (3) from rat small intestine. Ceramide was purchased from Analabs. Mono-, di-, tri- and tetrahexosylceramide were gifts from Dr. S.K. Kundu, purified from human erythrocytes (9).

Lipid Extraction

Six timed-pregnant Dorset sheep were obtained from Hidden Valley Farms (Reistertown, Maryland). The day after the mating night was taken as the first day of gestation. Animals were killed between 125-140 days later and the crown-rump length of each fetus determined to confirm gestational age. Colons (minus the cecum) were removed, washed and the mucosa scraped off with a glass slide. The mucosa was then homogenized in methanol and the lipids extracted using chloroform/methanol mixtures of increasing polarity (10).

Ganglioside and Glycolipid Purification

Total lipid extracts were partitioned with water and the lower phase washed four times with Folch upper phase (chloroform/methanol/water; 3:48:47, v/v/v) to insure complete extraction of gangliosides into the aqueous phase (10). Upper phase gangliosides were then purified by passage through Sep-Pak cartridges as described (11).

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The neutral glycolipids were purified by chromatography of the acetylated neutral lipids on a Florisil column (12). The gangliosides and deacetylated neutral glycolipids were analyzed by HPTLC on Silica-gel 60 precoated plates (Merck) in the solvent system chloroform/methanol/water/1% $CaCl_2$ (60:35:7:1, v/v/v/v) (13).

Individual glycolipids were quantified after scraping the silica-gel area corresponding to their position following migration. The amount of sphingoid bases liberated by methanolysis was determined by fluorimetry after reaction of the free bases with Fluorescamine (14). Quantitation of the various gangliosides was performed using a Beckman densitometer as described by Mullin et al (13).

Recovery

Known amounts of the ganglioside standard mixture and the neutral glycolipid/ceramide standard mixture were added to the mucosal samples and carried through the extraction and chromatographic procedures to determine recovery of the various glycosphingolipids and ceramide (15). Recoveries for GM_1, GM_2, GM_3, GD_{1a} and GT_{1a} were found to be 86, 84, 82, 74 and 66%, respectively. Recoveries for ceramide, mono-, di-, tri- and tetrahexosylceramide were 81, 80, 90, 78 and 79%, respectively.

Neuraminidase Treatment of Gangliosides

Purified gangliosides were dried under nitrogen, reconstituted in 100 µl of water and 100 µl of neuraminidase (*V. cholerae*, Behring Diagnostics) was added. The samples and controls were incubated at 37 C for 15 hr. The reaction was terminated by the addition of 20 vol of chloroform/methanol (2:1, v/v), dried under nitrogen, and reconstituted in 100 µl of chloroform/methanol (2:1, v/v). N-glycolyl and N-acetylneuraminic acids were separated on HPTLC plates using a solvent system of n-propanol/water/ammonium hydroxide (60:28:1.5, v/v/v) (16) and visualized with resorcinol.

Analysis of Carbohydrate Moieties of Gangliosides and Neutral Glycolipids

Carbohydrate moieties were identified by gas-liquid chromatographic (GLC) analysis of their trimethylsilyl derivatives as described (3,17).

Analysis of Fatty Acids and Long Chain Bases of Glycosphingolipids

Individual glycosphingolipids were hydrolyzed in 1 ml of dry methanolic HCl (0.75 M) at 80 C for 16 hr, followed by extraction of fatty acid methyl esters by hexane (18). The methanolic phase was then alkalinized with aqueous NaOH and the long chain bases extracted by diethyl ether (19).

Nonhydroxy and hydroxy fatty acids were separated by chromatography on a Florisil column as described (20). In some experiments, a known amount of C_{21} fatty acid methyl ester was added as an internal standard to quantitate the nonhydroxy and hydroxy fatty acids in the samples (5). Nonhydroxy fatty acid methyl esters were dissolved in a small volume of hexane, while hydroxy fatty acid methyl esters were silylated prior to chromatography (5). Analyses of both nonhydroxy and hydroxy fatty acids were performed on a Hewlett-Packard 3390A integrator as described (5). Peak identification was based on identical retention times with known standards run under the same conditions (5).

Long chain bases, free from fatty acids, were dissolved in chloroform, washed with 0.1 M NaOH and then washed repeatedly with water. The chloroform phase was dried under N_2, and the residue silylated as described (21). The long chain bases were then analyzed by GLC on an OV-1 column operated with temperature programming from 245 to 305 C at 3 C per min (5). Identification was based on identical retention times with known standards run under the same conditions (5).

RESULTS AND DISCUSSION

The present data indicate that qualitative and quantitative differences in glycosphingolipid and ceramide exist between fetal and adult sheep colonic mucosa. The results of the analysis of the content and relative percentages of the gangliosides, neutral glycosphingolipids and ceramide by HPTLC are summarized in Table 1. As shown in this table, the total content of gangliosides was approximately 30% lower in adult than fetal mucosa, whereas adult tissue contained about twice as much neutral glycolipid and ceramide as fetal tissue.

The major gangliosides of fetal and adult sheep colonic mucosa, as assessed by HPTLC, were in descending order, GD_{1a}, GM_3 and GM_2 (Table 1). Adult colonic, but not fetal, mucosa also possessed a ganglioside that had similar mobility to GT_{1a} (Table 1). Recently, the ganglioside composition of adult large intestine was described (22). In that species, GM_3 and not GD_{1a} was found to be the predominant ganglioside in this organ.

Analysis of the sugar moieties of the major gangliosides of fetal and adult colonic mucosa by GLC (Table 2) revealed that: (i) GM_3 contained equimolar ratios of glucose, galactose and sialic acid; (ii) GM_2 contained equimolar

TABLE 1

Content and Relative Percentage of Gangliosides, Neutral Glycosphingolipids and Ceramide from Adult and Fetal Sheep Colonic Mucosa[a]

Gangliosides					Neutral glycosphingolipids and ceramide				
	Adult (nmoles NeuAc/ mg protein)	%	Fetus (nmoles NeuAc/ mg protein)	%		Adult (nmoles sphingosine mg protein	%	Fetus (nmoles sphingosine/ mg protein	%
GM_3	41.7	22.3	113.3	41.2	Ceramide	662.5	62.5	338.6	62.7
GM_2	33.1	17.7	34.4	12.5	Monohexosylceramide	133.6	12.6	58.3	10.8
					Dihexosylceramide	44.5	4.2	–	–
GD_{1a}	94.8	50.7	130.0	47.5	Trihexosylceramide	68.9	6.5	–	–
GT_{1a}	20.2	10.8	–	–	Tetrahexosylceramide	155.8	14.7	137.7	25.5
	189.8		277.7			1065.3		534.6	

[a]Values represent means of 3 separate preparations. S.E. were less than 10% of the mean values.

TABLE 2

Analysis of Sugar Moieties in Gangliosides and Neutral Glycosphingolipids from Adult and Fetal Sheep Colonic Mucosa[a]

	Adult		Fetus	
	Sugar	Molar ratios	Sugar	Molar ratios
Monohexosylceramide	Gal/Glc	0.2/0.8	Gal/Glc	0.2/0.8
Dihexosylceramide	Gal/Glc	1.1/1.0	–	–
Trihexosylceramide	Gal/Glc	2.2/1.0	–	–
Tetrahexosylceramide	Gal/Glc/GalNAc	2.0/1.1/0.9	Gal/Glc/GalNAc	2.1/1.2/1.0
GM_3	Glc/Gal/SA	1.0/1.2/1.0	Glc/Gal/SA	0.9/1.0/1.1
GM_2	Glc/Gal/GalNAc/SA	1.1/0.9/1.0/1.0	Glc/Gal/GalNAc/SA	1.0/1.0/0.9/1.1
GD_{1a}	Glc/Gal/GalNAc/SA	1.0/2.1/1.0/2.0	Glc/Gal/GalNAc/SA	1.0/2.0/1.0/2.0
GT_{1a}	Glc/Gal/GalNAc/SA	1.1/2.0/1.1/3.1	–	–

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

ratios of N-acetylgalactosamine, galactose, glucose and sialic acid; (iii) GD_{1a} contained 1 mol of N-acetylgalactosamine, 1 mol of glucose and 2 moles each of sialic acid and galactose, and (iv) GT_{1a} (adult mucosa) contained 1 mol of N-acetylgalactosamine, 1 mol of glucose, 2 moles of galactose and 3 moles of sialic acid. This further established their identities.

As demonstrated in Table 3, after treatment with neuraminidase, the sialic acid moieties of the gangliosides of both fetal and adult colonic mucosa contained N-acetyl and N-glycolyl forms. The major sialic acid moiety of GM_3 in all samples was N-acetylneuraminic acid, whereas GM_2 and GD_{1a} (fetal and adult) and GT_{1a} (adult) contained approximately equal amounts of the two forms.

In agreement with recent studies in rat adult large intestine (22), mono- and tetrahexosylceramide were found to be the principal neutral glycolipids in sheep adult mucosa (Table 1). Smaller amounts of di- and trihexosylceramide were also present in adult mucosa. Mono- and tetrahexosylceramide were the major neutral glycosphingolipids in fetal large intestine as well. However, this tissue lacked di- and trihexosylceramide (Table 1). Ceramide, a non-sugar containing compound, was a major component of both fetal and adult mucosal lipids (Table 1). Analysis of the sugar moieties of the neutral glycolipids of fetal and adult sheep colonic mucosa by GLC further established their identities (Table 2).

In order to further elucidate differences between fetal and adult glycosphingolipids, the fatty acids and long chain bases of the major gangliosides, GD_{1a} and GM_3, and the major neutral glycosphingolipids, mono- and tetrahexosylceramide, were extracted and examined. All fetal and adult glycosphingolipids examined

TABLE 3

Relative Percentage of N-Acetyl and N-Glycolylneuraminic Acid in Gangliosides of Fetal and Adult Sheep Colonic Mucosa[a]

	Adult		Fetus	
	N-acetyl	N-glycolyl	N-acetyl	N-glycolyl
GM_3	69.5 ± 2.5	30.5 ± 2.1	69.1 ± 1.2	31.0 ± 1.2
GM_2	54.6 ± 4.2	45.3 ± 4.8	58.6 ± 1.2	41.0 ± 1.6
GD_{1a}	40.5 ± 2.1	59.2 ± 2.3	45.1 ± 1.7	54.2 ± 2.6
GT_{1a}	49.3 ± 2.6	50.5 ± 3.0	–	–

[a]Values represent means ± SE of 3 separate preparations.

TABLE 4

Percentage of Hydroxylated Fatty Acids Relative to Total Fatty Acids of Glycolipids Extracted from Adult and Fetal Sheep Colonic Mucosa[a]

	Adult colonic mucosa	Fetal colonic mucosa
Ceramide	67.1 ± 4.1	29.7 ± 4.7
Monohexosyl-ceramide	64.2 ± 3.9	32.6 ± 4.1
Tetrahexosyl-ceramide	69.4 ± 6.2	40.1 ± 5.4
GM_3	72.1 ± 4.6	36.3 ± 3.6
GD_{1a}	67.4 ± 3.8	31.2 ± 4.2

[a]Values represent means ± SE of 3 separate preparations and were obtained with the use of methyl heneicosanoate as internal standard.

contained both hydroxy and nonhydroxy fatty acids. As shown in Table 4, however, hydroxy fatty acids accounted for only 30-40% of total fatty acids present in fetal glycosphingolipids, whereas hydroxy fatty acids accounted for 60-70% of the total fatty acids of adult glycosphingolipids. While the glycosphingolipid composition of fetal fat colon has not yet been reported, prior studies in fetal and adult rat small intestine have demonstrated that α-hydroxylation of fatty acids of glycolipids occurred only during postnatal development (6). In sheep colonic mucosa, however, since hydroxy fatty acids were present in fetal glycolipids, either α-hydroxylase activity was present in this mucosa or, less likely, the hydroxy fatty acids originated from the mother. Further

TABLE 5

Fatty Acid Composition of GM_3 and Monohexosylceramide from Adult and Fetal Sheep Colonic Mucosa[a]

Fatty acids	GM_3				Monohexosylceramide			
	Adult		Fetus		Adult		Fetus	
	HFA %	NFA %	HFA %	NFA %	HFA %	NFA %	HFA %	NFA %
16:0	trace	9.9	trace	19.3	14.2	11.3	3.6	trace
16:1	trace	trace	trace	trace	trace	2.1	trace	trace
18:0	8.8	37.2	2.4	trace	2.4	26.9	36.8	43.7
18:1	2.7	31.7	trace	40.9	4.4	48.6	38.7	14.7
18:2	trace	trace	trace	22.3	trace	trace	4.3	trace
20:0	–	–	trace	trace	3.2	trace	trace	3.7
20:1	–	–	–	trace	trace	trace	trace	–
20:2	–	–	trace	trace	6.8	trace	–	–
20:4	–	–	trace	trace	trace	trace	–	–
22:0	trace	5.8	trace	3.4	–	trace	4.3	10.7
22:1	–	3.1	trace	trace	3.8	trace	trace	trace
24:0	16.1	3.8	3.4	trace	3.3	–	3.3	12.9
26:0	9.9	3.9	81.8	7.6	45.2	–	2.5	trace
28:0	15.6	4.4	5.5	–	8.5	–	–	–
30:0	44.7	–	–	–	12.2	–	–	–

HFA=Hydroxy fatty acids; NFA= nonhydroxy fatty acids.

[a]Values are means of 3 separate preparations. SE were all less than 10% of the mean value. Trace indicates 2.0% or less of the total.

TABLE 6

Percentage of Phytosphinosine in the Sphingoid Bases of Glycosphingolipids of Sheep Adult and Fetal Colonic Mucosa[a]

Glycolipid	Adult	Fetus
Monohexosylceramide	31.9	5.5
Tetrahexosylceramide	61.3	16.8
GM_3	34.1	17.0
GD_{1a}	60.0	40.0

[a]Each figure represents the mean of 2-4 determinations of the aldehydes by gas-liquid chromatography.

studies will be necessary to clarify this issue, but the present findings support the concept that the pattern of glycosphingolipid composition is species-specific and tissue-specific (1).

Cerotic (26:0) and stearic (18:0) acids were the predominant hydroxy and nonhydroxy fatty acids, respectively, of fetal and adult GD_{1a}. As shown in Table 5, however, the predominant hydroxy and nonhydroxy fatty acids, respectively, of adult GM_3 were melissic (30:0) and stearic acids, whereas in fetal GM_3 they were cerotic and oleic (18:1) acids. In general, the nonhydroxy fatty acids of fetal GM_3 were more unsaturated than their adult counterparts.

Analysis of the fatty acids of ceramide, mono- and tetrahexosylceramide by gas-liquid chromatography revealed that the major hydroxy fatty acids of fetal and adult mucosal ceramide were cerotic, montanic (28:0) and melissic acids, while the major nonhydroxy fatty acids were stearic and palmitic (16:0) acids. The principal hydroxy fatty acids of tetrahexosylceramide in fetal and adult mucosa were lignoceric (24:0) and montanic acids, respectively, while the principal nonhydroxy fatty acid of this fetal mucosal glycosphingolipid was cerotic acid and, of the adult, montanic acid. As shown in Table 5, the major nonhydroxy fatty acids of both fetal and adult monohexosylceramide were oleic and stearic acids. Oleic and stearic acids were the principal hydroxy fatty acids of monohexosylceramide in fetal tissue, while cerotic acid was the major hydroxy fatty acid in adult mucosa.

In most mammalian tissues, the ceramide backbone of glycosphingolipids is sphingosine (C18-sphinganine) (8). In some tissues, however, such as kidney and small intestine (23-25), the major base is phytosphingosine (4D-hydroxysphinganine). In adult rat colon the predominant long chain base of epithelial cell glycolipids also was demonstrated recently to be phytosphingosine (23). As shown in Table 6, while this was true for GD_{1a} and tetrahexosylceramide of adult sheep colonic mucosa, sphingosine was the major long chain base of GM_3 and monohexosylceramide in this tissue. In agreement with recent studies in rat intestine (9), however, the relative percentage of phytosphingosine was higher in adult than fetal tissue in all glycosphingolipids examined in the sheep colon (Table 6). These results suggest that sheep colon may acquire an increased capacity for synthesizing phytosphingosine at some time between late fetal development (125-140 days) and adulthood. It seems reasonable to suggest that the difference in glycosphingolipids of fetal and adult sheep colonic mucosa have functional significance and that further studies of these substances should clarify the questions of function and the mechanisms for maintaining the difference in glycosphingolipid composition.

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Further Studies on Sphingolipids in Wheat Grain

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ABSTRACT

The principal molecular species of sphingolipids in wheat grain were confirmed to be *N*-2′-hydroxylignoceroyl-4-hydroxysphinganine for ceramide, and 1-*O*-*β*-glucosyl-, 1-*O*-[*β*-mannosyl(1→4)-*O*-*β*-glucosyl]-, 1-*O*-[*β*-mannosyl(1→4)-*O*-*β*-mannosyl(1→4)-*O*-*β*-glucosyl]- and 1-*O*-[*β*-mannosyl(1→4)-*O*-*β*-mannosyl(1→4)-*O*-*β*-mannosyl(1→4)-*O*-*β*-glucosyl]-*N*-2′-hydroxypalmitoyl(or hydroxyarachidoyl)-*cis*-8-sphingenine for mono-, di-, tri- and tetraglycosylceramide, respectively. A novel glycolipid, cellobiosylceramide, was found as the minor diglycosylceramide; the major species was characterized to be 1-*O*-[*β*-glucosyl(1→4)-*O*-*β*-glucosyl]-*N*-2′-hydroxypalmitoyl (or hydroxyarachidoyl)-*cis*-8-sphingenine. It was observed in these sphingolipids that the dihydroxy bases were combined mainly with C_{16} and C_{20} acids, whereas the trihydroxy bases combined mostly with acids of chain length of 20 or more.
Lipids 20:337-342, 1985.

INTRODUCTION

Although the fundamental structures of wheat sphingolipids were reported by Laine and Renkonen (1) and the present authors (2), their chemical characterization has not yet been carried out completely. In this paper we describe final establishment of the principal molecular species of CM and G_{1-4}CM, isolation of cellobiosylceramide, a minor species of G_2CM, and presence of the two major patterns of the ceramide moiety in wheat sphingolipids.

EXPERIMENTAL METHODS

Isolation and Fractionation of Sphingolipids

Sphingolipids were separated and purified according to the procedures described previously (2). Wheat grain was extracted with chloroform-methanol and water-saturated butanol, and the total lipids obtained were subjected to silicic acid column chromatography, mild alkaline hydrolysis and preparative silica gel TLC to isolate CM, G_1CM, G_2CM, G_3CM and G_4CM. G_2CM was further fractionated into mannosylglucosyl- and diglucosylceramide by silica gel-borax (95:5, w/w) TLC with chloroform-methanol-water (65:25:4, v/v/v).

Compositional Analyses of Constituents

Constituents (fatty acid, sugar and sphingoid) of sphingolipids and their partially deglycosylated products were liberated by acid degradation and analyzed mainly by GLC (2). After separation into dihydroxy and trihydroxy base-containing species of CM and G_1CM by borax-containing silica gel TLC, the two groups were methanolyzed to determine the respective fatty acid composition by GLC (3,4).

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Structural Analyses of Glycosidic Chain

Anomeric configurations of the glycosidic linkages were analyzed by PMR at 100 C using deuterium pyridine as solvent (5). The analysis was performed on a JEOL JNM-FX-200 (200 MHz) instrument, and the chemical shift was expressed in δ value (ppm) using tetramethylsilane as the internal standard. Moreover, the sugars of acetylated glycolipids surviving oxidation with chromium trioxide were examined to determine the anomeric configuration (6).

Binding positions of the glycosidic chains were assayed by GLC and GC-MS (7) of the methylated methylglycosides, which were obtained by permethylation of glycolipids and purification by silica gel TLC with chloroform-benzene-acetone (80:20:10, v/v/v) followed by methanolysis, as described previously (2).

Analyses of Molecular Species

Molecular species were analyzed by FD-MS and GC-MS. FD-MS was recorded on a JEOL JMS-01SG instrument. The emitter current was programmed from 10 to 20mA at the rate of 5mA per min. GC-MS was performed on a Hitachi RMU-6MG instrument under the same conditions as described previously (3,4).

RESULTS

β-Configuration of Glycosidic Linkage

PMR of G_{1-3}CM is shown in Figure 1. The anomeric protons of the glucose residue attached to the ceramide moiety in G_{1-3}CM gave the doublet at 4.77 ppm (J=7.0Hz), 4.77 ppm (J=7.4Hz) and 4.76 ppm (J=7.4Hz), which corresponded to the chemical shift (near 4.4 ppm) and coupling constant (J=7.2±0.2Hz) indicating the *β*-glycosidic linkage but not the *α*-one (5.12

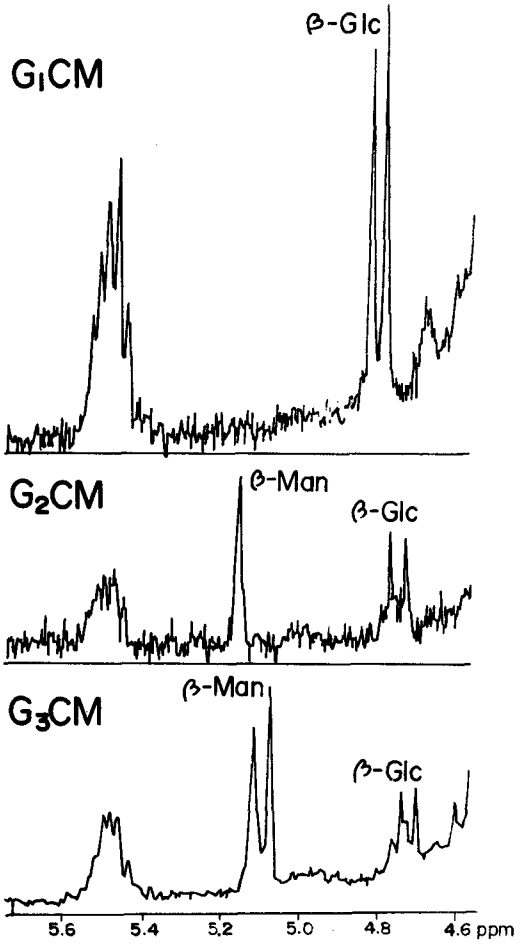

FIG. 1. PMR (200 MHz) of glycosylceramides in wheat grain.

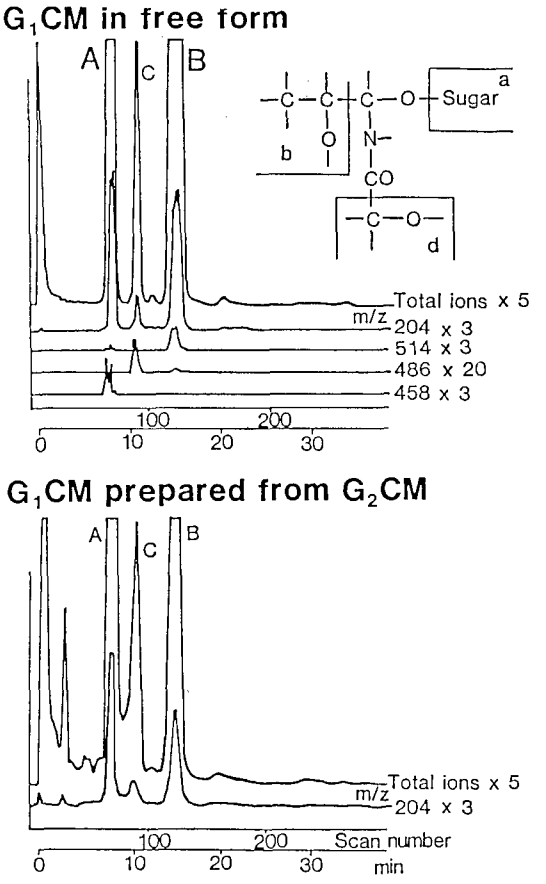

FIG. 2. Mass chromatograms of G_1CM in wheat grain. Trimethylsilyl ether derivatives of G_1CM were analyzed by GC-MS with datalizer. Column temperature was 320 C. The energy level of the ion source was 20 eV and the ionizing current 80 μA. Ions at m/z 458, 486 and 514 were the fatty acid fragment (M−a−b+73) due to hydroxypalmitic, hydroxystearic and hydroxyarachidic acids, respectively, and that at m/z 204 originating from the sugar moiety was commonly detected in all the G_1CM peaks. The numerals on the right show the amplification of the intensity.

±0.33 ppm, J=3.2±0.6Hz) (5.8). The signal due to the anomeric proton of the mannose residue in G_2CM was recognized at 5.13 ppm, and those in G_3CM at 5.15 and 5.10 ppm. These chemical shifts approximated that of the anomeric proton of mannose with β-linkage (near 5.2 ppm), but not with α-linkage (near 5.8 ppm) (9,10). The nuclear magnetic resonance spectrum of G_4CM was not measured due to the small amount. The survival component sugars in acetylated glycolipids after oxidation with chromium trioxide were less than 17%, as reported previously (2).

Principal Molecular Species

Mass chromatograms of G_1CM in the free form and G_1CM prepared from G_2CM by partial degradation are shown in Figure 2 (3). Both the chromatograms were nearly identical with each other. When the mass spectra of major peak A and B were assayed (11,12), the sphingoid fragment ion was commonly recognized at m/z 311(b) due to 8-sphingenine. As the fatty acid fragments (M−a−b+73 and d), ions at m/z 458 and 299 due to hydroxypalmitic acid and those at m/z 514 and 355 derived from hydroxyarachidic acid were found in the spectra of peaks A and B, respectively. Based on these ions as well as fragment ions originated from the ceramide moiety (4), peaks A and B were identified as 1-*O*-glucosyl-*N*-2′-hydroxypalmitoyl-8-sphingenine and 1-*O*-glucosyl-*N*-2′-hydroxyarachidoyl-

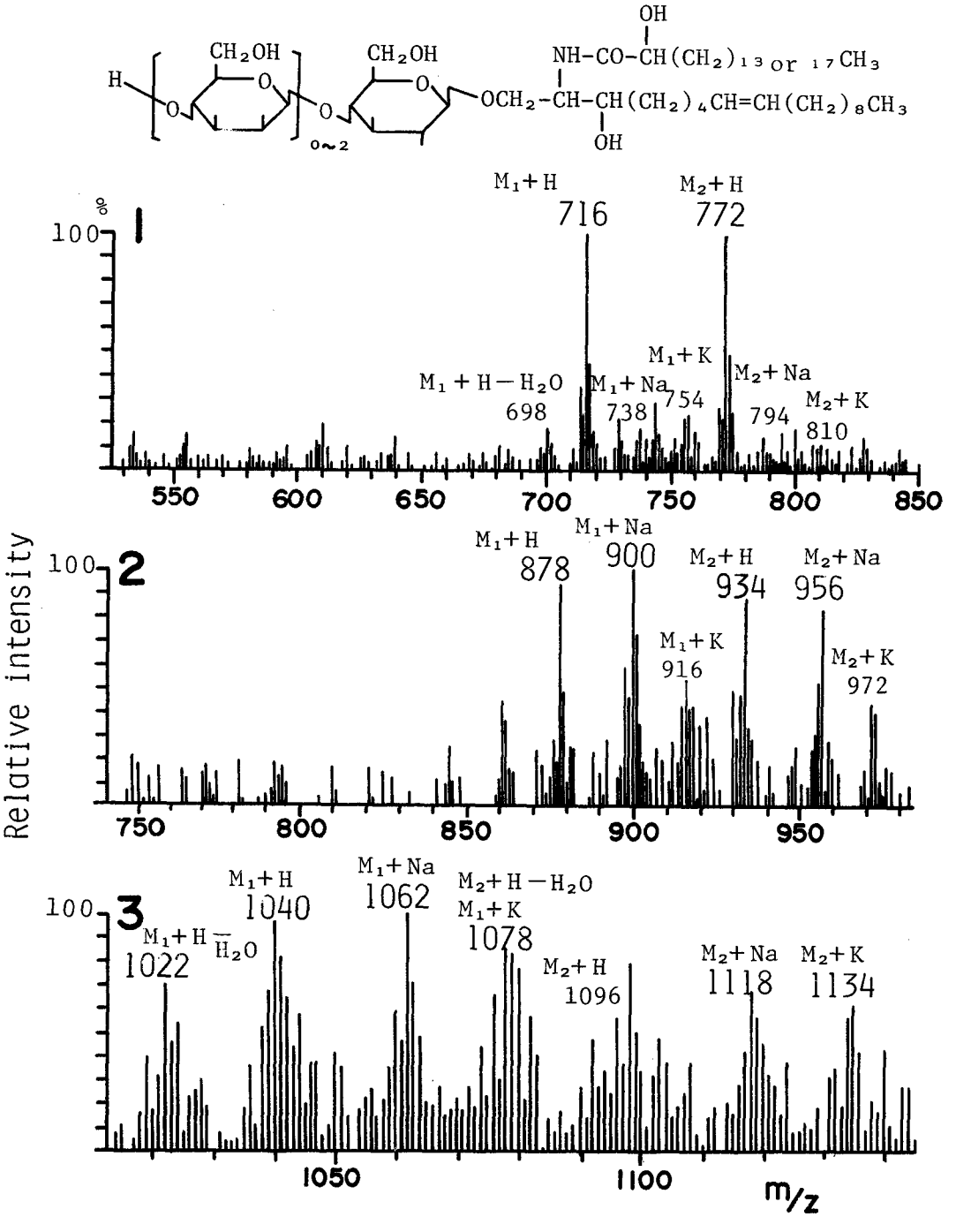

FIG. 3. FD-MS of glycosylceramides in wheat grain. 1, G_1CM; 2, G_2CM and 3, G_3CM. The component ceramide species of M_1 and M_2 in the spectra are commonly 2-hydroxypalmitoyl-8-sphingenine and 2-hydroxy-arachidoyl-8-sphingenine, respectively.

8-sphingenine, respectively. Likewise, minor peak C was characterized as G_1CM composed mainly of 2-hydroxystearic acid and 8-sphingenine.

FD-MS of $G_{1-3}CM$ is shown in Figure 3. As M+H ions demonstrating molecular weight (13) were found at m/z 716 (the base peak) and 772 in the spectrum of G_1CM, the principal species were confirmed to have the ceramide moieties, in which 8-sphingenine was combined with hydroxy acids of C_{16} or C_{20}. FD-MS of G_2CM and G_3CM exhibited a remarkable ion due to M+Na (13). The ions derived from the species composed of 8-sphingenine and C_{16} hydroxy acids were observed at m/z 900 in G_2CM and at m/z 1062 in G_3CM, respectively, both ions being the base peak. Moreover, the M+Na ions originating from the species having 8-sphingenine and C_{20} hydroxy acid were detected notably at m/z 956 and 1118 in the spectra of G_2CM and G_3CM, respectively. The result supported these species belong to the major types.

Detection of Diglucosylceramide

By the preparative TLC on silica gel-borax of G_2CM prepared from wheat grain, two lipid species, X (Rf 0.33) and Y (Rf 0.43) were obtained in an approximate ratio of 9:1. IR spectra of the two lipids agreed with that of whole G_2CM. As the component sugars, glucose and mannose were detected in an approximate ratio of 1:1 for X, whereas there was only glucose for Y (Table 1). This implies that X corresponded to mannosylglucosylceramide and Y to diglucosylceramide.

Structure of Diglucosylceramide

GLC of methylated methyl glycosides prepared from permethylated mannosylglucosylceramide and diglucosylceramide are shown in Figure 4. From mannosylglucosylceramide methyl 2,3,4,6-tetra-*O*-methylmannoside and

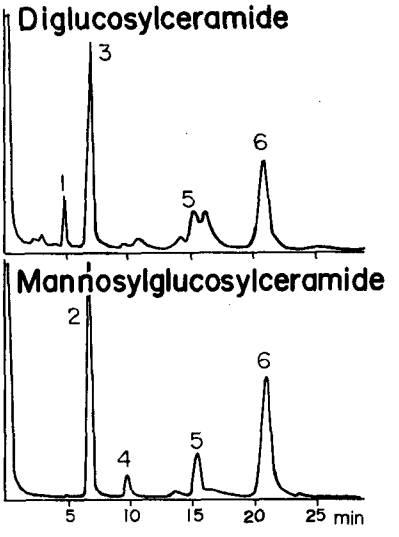

FIG. 4. GLC of permethylated methyl glycosides from G_2CM in wheat grain. A glass column, 0.3 × 200 cm, was packed with 3% NPGS, and the column temperature was 155 C. 1 and 3, β- and α-methyl 2,3,4,6-tetra-*O*-methylglucosides; 2 and 4, β- and α-methyl 2,3,4,6-tetra-*O*-methylmannosides; 5 and 6, β- and α-methyl 2,3,6-tri-*O*-methylglucosides.

methyl 2,3,6-tri-*O*-methylglucoside were obtained as reported previously (2). In analysis of the methyl glycosides from diglucosylceramide, the internal glucose with 1→4 bond was recognized in addition to the terminal glucose. When the acetyl derivatives of both the G_2CM were oxidized with chromium trioxide, the recovery of the component sugars was generally low (Table 1). This suggests that glucose and mannose residues all were linked with β-glycosidic configuration. These results demonstrated that not only the major G_2CM with the Man(β1→4)-Glc(β1→1′)-chain previously reported but also the minor G_2CM species with the sugar moiety

TABLE 1

Sugars and Their Ratios (%) in Partially Methanolyzed Products, and Chromium Trioxide Oxidation of G_2CM in Wheat Grain

		Fractions obtained from methanolyzates		
G_2CM	Component sugar	Lipid-free sugar	G_1CM	G_2CM
Mannosylglucosyl type (X)	Glucose	10.7	92.5	51.7 (14.0)[a]
(Rf 0.33)	Mannose	89.3	7.5	48.3 (5.3)
Diglucosyl type (Y)	Glucose	96.8	96.7	96.3 (19.1)
(Rf 0.43)	Mannose	3.2	3.3	3.7 (7.5)

[a]Ratio of oxidation-resistant sugar to original sugar.

of Glc(β1→4)-Glc(β1→1′)-,cellobiosylceramide, were present in wheat grain. The composition of the ceramide moiety in cellobiosylceramide was similar to those in mannosylglucosylceramide and whole G_2CM (2).

Distribution of Hydroxy Acids in Sphingolipids

Table 2 shows hydroxy acid composition in the dihydroxy base- and trihydroxy base-containing species of CM, G_1CM and G_2CM. As a general tendency, the principal acids in the dihydroxy base-containing species were C_{20} and C_{16} ones, and those in the trihydroxy base-containing species C_{24} and C_{20} ones.

TABLE 2

Composition of 2-Hydroxy Fatty Acids Based on Sphingoid Type in CM, G_1CM and G_2CM from Wheat Grain (%)

2-Hydroxy fatty acid	CM		G_1CM		G_2CM	
	D[a]	T[b]	D	T	D	T
16:0	29.5	4.2	37.8	19.5	39.9	18.2
18:0	2.1	0.5	8.3	2.2	5.3	3.3
20:0	60.5	10.4	49.5	14.9	37.3	26.8
22:0	0.6	5.4	1.9	11.7	3.6	7.7
23:0	1.9	4.4	< 0.1	2.7	2.3	6.6
24:0	5.4	64.1	0.6	33.9	5.5	22.0
24:1	<0.1	1.6	0.3	2.0	1.0	5.1
25:0	<0.1	6.9	–	3.7	0.2	0.9
26:0	<0.1	2.4	–	8.3	2.1	6.8
Others	<0.1	0.1	1.6	1.1	2.8	2.6

[a]D = Dihydroxy base-containing species.

[b]T = Trihydroxy base-containing species.

DISCUSSION

In wheat CM trihydroxy sphingoid was the major component (2) and C_{24} hydroxy acid was distributed overwhelmingly in the trihydroxy base-containing species (Table 2). The data supported that the principal species of CM was *N*-hydroxylignoceroyl-4-hydroxysphinganine, the structure of which had been reported previously (2). In this study the anomeric configurations of the glycosidic linkages in G_{1-3}CM, previously presumed on the basis of the oxidation data, were confirmed to be all with the β-glycosidic bond by high resolution PMR analyses. Although G_4CM was not analyzed, there is no doubt as to whether the four hexoses in G_4CM were all linked β-glycosidically, since effectiveness of the chromic acid oxidation method was substantiated as mentioned above. Other experimental evidence showed the principal component ceramide of G_{1-3}CM in wheat grain to be commonly *N*-hydroxypalmitoyl(or hydroxyarachidoyl)-8-sphingenine. Since the compositions of fatty acids and sphingoids in wheat G_{1-4}CM were similar to one another, the major ceramide of G_4CM is likely to be the same type as those of G_{1-3}CM. Therefore, the principal molecular species of G_{1-4}CM in wheat are 1-*O*-β-glucosyl-, 1-*O*-[β-mannosyl(1→4)-*O*-β-glucosyl]-, 1-*O*-[β-mannosyl(1→4)-*O*-β-mannosyl(1→4)-*O*-β-glucosyl]- and 1-*O*-[β-mannosyl(1→4)-*O*-β-mannosyl(1→4)-*O*-β-mannosyl(1→4)-*O*-β-glucosyl]-*N*-hydroxypalmitoyl (or hydroxyarachidoyl)-8-sphingenine, respectively.

In wheat G_2CM, the minor diglucosyl (cellobiosyl) type was found, as well as the major mannosylglucosyl one. The present data showed that the principal molecular species could be proposed as 1-*O*-[β-glucosyl(1→4)-*O*-β-glucosyl]-*N*-2′-hydroxypalmitoyl (or hydroxyarachidoyl)-8-sphingenine. The glycosidic structure of the G_2CM species was identical with that of cellobiosylceramide separated from rice for the first time. Previously we reported that G_{2-4}CM having not only the mannose but also the glucose residue at the terminal were present in rice grain (14). Therefore, it is possible that the minor $G_{3,4}$CM species with the glucose residue at the terminal are also existent in wheat grain.

In the present study hydroxy acid compositions in the dihydroxy base-containing species of wheat sphingolipids were shown to be different from those in the trihydroxy base-containing ones (Table 2). The same results have been obtained from analyses of the sphingolipids in spinach leaves (3), soybeans (4), rice (submitted for publication) and pea seeds (15). It may be general in plant sphingolipids that dihydroxy base is linked mainly to hydroxy acids with carbon chains of 16 or 20, while trihydroxy base is linked to those with 20 or more, especially 24.

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Evidence for Distinct Precursor Pools for Biliary Cholesterol and Primary Bile Acids in Cebus and Cynomolgus Monkeys

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ABSTRACT

The abnormal metabolism and distribution of plasma lipoproteins have been associated with atherosclerosis and gallstones. To better understand the process of cholesterol excretion, a study was designed to determine whether the contribution of lipoprotein free ^{14}C-cholesterol (as LDL or HDL) to biliary cholesterol or primary bile acids differs in two species of nonhuman primates, cebus and cynomolgus monkeys, having opposite plasma LDL/HDL ratios. Since amino acid conjugation might influence bile acid synthesis or secretion, the taurine and glycine conjugates of newly synthesized primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA), were measured in the species capable of conjugating with taurine or glycine (cynomolgus). After total bile acid pool washout, monkeys were infused with human LDL or HDL labeled with free ^{14}C-cholesterol, and the specific activities (SA) of biliary cholesterol and primary bile acid conjugates were determined. In both species, regardless of the lipoprotein infused, the SA of biliary cholesterol and CA were greater than those for total bile acids and CDCA, respectively. In cynomolgus, the SA of glycine conjugates was higher for CA than CDCA, while the SA of taurine conjugates was greater for CDCA than CA. Under these conditions, (i) infused lipoprotein free cholesterol (as either LDL or HDL) contributed more to biliary cholesterol than to bile acids and more to CA than to CDCA; (ii) glycine conjugated preferentially with CA rather than CDCA, while taurine was the preferred conjugate for CDCA. Further, whereas the two primary bile acids had similar rates of synthesis and turnover in cynomolgus, basal bile acid synthesis was much greater in cebus and the CDCA turnover appeared disproportionately large.
Lipids 20:343-349, 1985.

INTRODUCTION

Although the pathogenesis of atherosclerosis has been shown to be greatly influenced by disturbances in cholesterol metabolism (1), the impact of the latter on gallstone disease has been investigated widely, yet remains unclear (2,3). Epidemiological evidence reveals a positive correlation between low density lipoproteins (LDL) and atherosclerosis (1,4), whereas elevated levels of high density lipoproteins (HDL) are protective (5-7). Furthermore, the incidence of human cholesterol gallstones is increased among populations with a high incidence of atherosclerosis (8,9). Although the biochemical mechanisms underlying these major disturbances of lipid metabolism have been investigated (10,11), the exact relationship between these two diseases remains unclear. Because of the strong association between circulating plasma lipoprotein cholesterol and atherosclerosis on the one hand (1,4-7,11), and biliary sterol composition and gallstones on the other (12,13), one approach has been to study the precursor-product relationship between these sterol pools. This is accomplished by measuring the contribution of lipoprotein cholesterol versus newly synthesized cholesterol to biliary cholesterol and bile acids (14-18).

Schwartz and his coworkers compared the uptake of radio-labeled free cholesterol in LDL and HDL following injection of these lipoproteins in human patients with external biliary fistulas (15-17). They concluded that biliary cholesterol and bile acids are preferentially derived from HDL, compared to LDL, and that HDL free cholesterol is a more important donor than the lipoprotein cholesterol ester pool. Recently, Portman and his colleagues traced the incorporation of radio-labeled free and esterified cholesterol in LDL and HDL into biliary cholesterol of squirrel monkeys (18). Although their results agreed with those of Schwartz, only the latter addressed the conversion of lipoprotein cholesterol into bile acids, and then only to estimate preferred incorporation of LDL or HDL free cholesterol into a single bile acid, chenodeoxycholic acid (18). Even though these studies showed a preferred isotope exchange between HDL and biliary sterols, a net mass transfer of cholesterol between these two pools was not demonstrated.

Lipoprotein cholesterol and biliary sterols are further linked by the observations that diet-induced hypercholesterolemia is often associated with lithogenic bile (19). It is also noteworthy that chenodeoxycholic acid (CDCA) has been used clinically to dissolve radiolucent cholesterol stones in patients with cholelithiasis

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(20), and is postulated to operate through two possible mechanisms, either by enhancing the cholesterol-solubilizing capacity of bile to promote the dissolution of gallstones (21), or by feedback inhibition of hepatic cholesterol synthesis to decrease cholesterol concentration in bile (22). This feedback on cholesterol synthesis suggests a possible precursor-product relationship, i.e. that CDCA is derived largely from newly synthesized cholesterol. Whether the remaining primary bile acid, cholic acid (CA), is the preferred product of lipoprotein cholesterol has not been explored.

The conjugation of primary bile acids with either taurine or glycine may serve an important function in bile acid and cholesterol homeostasis (23,24). Hardison (25) and Sjovall (26) indicated that the extent of bile acid conjugation with taurine is determined largely by hepatic taurine status. Previously, we demonstrated that during taurine depletion the tauro-CDCA pool size remained constant while the tauro-CA pool shrank markedly and was replaced by glyco-CA (27). These observations suggested that taurine might have preferred access to the CDCA pool, thereby promoting conservation of both taurine and CDCA during whole body taurine depletion. Whether glycine has preferred access to the CA pool has not been determined.

This study was designed to determine the characteristics of the precursor pools of cholesterol entering the liver via the lipoprotein route (infused as LDL or HDL) that give rise to biliary cholesterol and primary bile acids in two primate species, cebus and cynomolgus monkeys, with opposite LDL/HDL ratios. Furthermore, since the cynomolgus monkey conjugates bile acids with both taurine and glycine, we determined the preferential access of either conjugating amino acid to CA and CDCA synthesized from the injected lipoprotein cholesterol.

MATERIALS AND METHODS

Animals and Diet

Eight newborn cebus *(Cebus albifrons)* (four males and four females) and eight newborn cynomolgus *(Macaca fascicularis)* (four males and four females) were used in these studies. They were raised in nursery facilities at the New England Regional Primate Research Center (Southborough, Massachusetts). All monkeys were fed from birth a soy-protein commercial infant formula (Isomil®, Ross Laboratories, Columbus, Ohio) for 20 weeks before studies commenced. Blood samples were obtained at birth and at 4-week intervals to establish plasma cholesterol values.

Isolation and Labeling of ^{14}C-Cholesterol Lipoproteins

A blood sample was obtained from a normocholesterolemic fasting human donor in 15% Na_2EDTA, and plasma was separated by centrifugation for 20 min at 2500 rpm and 4 C. The density of plasma then was adjusted at 1.21 g/ml by adding solid KBr, after which plasma was overlayered with three consecutive solutions of NaN_2 and KBr of densities 1.063, 1.019 and 1.006. Plasma samples were then spun in a SW41 rotor using a Beckman L5-50 ultracentrifuge (Beckman Instruments, Palo Alto, California) at 37,000 rpm and 15 C for 44 hr (28). LDL and HDL bands were readily identified and carefully separated from the density ranges of 1.019-1.063 and 1.063-1.21, respectively.

The lipoprotein was labeled by incubation with a Whatman #1 filter paper impregnated with 10-20μCi of [4-^{14}C]-cholesterol for 4 hr at 5 C. This resulted in free exchange of ^{14}C-cholesterol with the lipoprotein free cholesterol moiety. The labeled lipoproteins were dialyzed overnight against 0.9% NaCl-0.01% EDTA at pH 7.4, and passed through a 0.22μm millipore filter for removal of particulate ^{14}C-cholesterol and sterilization prior to injection into monkeys. Analysis of these lipoproteins revealed: (i) typical homogeneous electrophoretic mobility for LDL and HDL on cellulose acetate (29), and (ii) the label was essentially all in the free cholesterol moiety (>90%) (30).

Experimental Procedure

After 5 mo of feeding, monkeys were fasted overnight and anesthetized with 30 mg/kg body weight of pentobarbital (Nembutal®, Abbott Laboratories, North Chicago, Illinois). A midventral incision was made and the biliary tract exposed. The cystic duct was ligated and the gallbladder contents aspirated and frozen in chloroform:methanol (2:1) for later analysis. The enterohepatic circulation was interrupted by cannulation and ligation of the common bile duct using a polyethylene (PE 50) cannula. Washout of the bile acid pool for estimation of the total pool size was enhanced by portal vein infusion of synthetic cholecystokinin-octapeptide (Kinevac®, Squibb, Princeton, New Jersey) at a level of 0.02μg/kg body weight/hr for 4 hr (31,32). Bile samples were collected at 30-min intervals for 4½ hr and frozen in chloroform:methanol (2:1) for later analysis.

When bile acid pool washout was complete (4½ hr) and the secretion rate of bile acids had reached a steady plateau equivalent to the hepatic synthesis rate (33), a dose of ^{14}C-cholesterol-labeled LDL or HDL was infused in the portal vein of the monkey at a rate calcu-

lated to be approximately twice the production rate of hepatic cholesterol. Based on an estimated daily cholesterol production of 100 mg per kg body weight (34), the infusion rate of human lipoprotein was adjusted to deliver 8.33 mg/kg/hr. Infusion lasted for 2 hr during which time bile samples were collected.

During the infusion, anesthesia was maintained with intramuscularly administered phencyclidine hydrochloride (Sernalyn®, Bioceutic Laboratories, St. Joseph, Missouri) because barbiturates are known to stimulate bile acid synthesis (35). In addition, the monkey was placed on a heating pad to maintain constant body temperature.

Biochemical Analysis

Bile samples were extracted in chloroform: methanol (2:1) according to Folch et al. (36), where bile acids were recovered in the methanol layer and cholesterol and phospholipids in the chloroform layer. Each solvent layer was aspirated into a separate tube, filtered to remove any solid particulates and counted in Aquasol® (New England Nuclear, Boston, Massachusetts) using a Beckman liquid scintillation counter. Total cholesterol was determined by the single reagent ferric perchlorate method of Wybenga et al. (37); phospholipids were assessed by the phosphorus method of Bartlett et al. (38), and total bile acids were measured by the enzymatic method of Admirand et al. (39) as validated by Turley and Dietschy (40). The washout technique of Dowling et al. (41) was used to calculate bile acid pool size. This method estimates pool sizes comparable to those obtained by isotope dilution methods (42). The concentration of individual bile acid conjugates in gallbladder samples was measured by high-performance liquid chromatography utilizing a modification of the method of Bloch and Watkins (43) and a Beckman Instruments (Fullerton, California) model 330 isocratic chromatograph. Bile acids were separated on an ultrasphere-ODS Altex® column (5μm 46 mm x 250 mm) (Palo Alto, California) and detected with Hitachi (Tokyo, Japan) model 110-40 variable wavelength spectrophotometer. The specific activity of primary bile acids in newly synthesized bile was calculated after mass and radiolabel determination following their separation on thin layer chromatography using n-butanol:acetic acid:water (10:1.1:1.1, v/v/v) for taurine conjugates and isoamyl acetate:propionic acid:n-propanol:water (4:3:2:1, v/v/v/v) for glycine conjugates (44). Plasma total, LDL and HDL cholesterol were determined following heparin-$MnCl_2$ precipitation of apoE, B-containing lipoproteins (45).

Statistical Analysis

Differences in specific activities between biliary cholesterol and individual bile acids within monkeys of the same treatment group were tested for statistical significance by paired t-test. Differences in specific activity of biliary sterols between monkey species and between injected lipoproteins were tested for significance by Student's t-test (46).

RESULTS

LDL/HDL Profiles

Although cebus and cynomolgus monkeys had similar concentrations of plasma total cholesterol, cebus monkeys had a predominantly HDL profile while the LDL cholesterol pool was larger in cynomolgus monkeys (Table 1).

Pool Size and Basal Synthesis of Bile Acids

Total biliary diversion in these two species of infant monkeys allowed complete washout of the bile acid pool. Bile acid secretion reached basal levels within 4½ hr of cannulating the common bile duct (Fig. 1). In the cebus mon-

TABLE 1

Plasma Total, LDL and HDL Cholesterol of 5-month old Cebus and Cynomolgus Monkeys

Parameter	Cebus (mg/dl)	Cynomolgus (mg/dl)
Total	155 ± 8	148 ± 14
LDL	56 ± 6[a]	98 ± 11
HDL	99 ± 3[a]	50 ± 6
LDL/HDL	0.55 ± 0.04[a]	2.09 ± 0.24

Values are mean ± SEM; 8 monkeys per group.
[a]Significantly different from cynomolgus ($P<0.001$, Student's t-test).

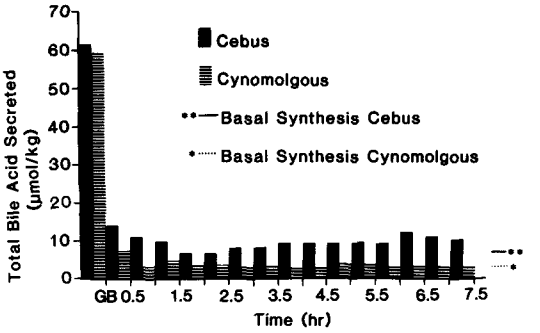

FIG. 1. Bile acid washout curves for cebus and cynomolgus monkeys indicate that bile acid secretion had achieved a steady, basal rate by 4½ hr after interruption of the enterohepatic circulation. At this time LDL or HDL free ^{14}C-cholesterol was infused into the portal vein of the monkey for 2 more hr. Basal synthesis in cebus (**) was significantly greater than that in cynomolgus (*).

TABLE 2

Pool Size and Basal Synthesis of Total Bile Acids, Cholic (CA), and Chenodeoxycholic Acids (CDCA), and Biliary Cholesterol Secretion in Infant Cebus and Cynomolgus Monkeys

	Bile acids						
	Pool size (μmol·kg^{-1})			Basal synthesis (μmol·kg^{-1}·hr^{-1})			Cholesterol secretion
Species	Total	CA	CDCA	Total	CA	CDCA	
Cebus	81 ± 6	58 ± 4[a,b]	16 ± 1[b]	13.7 ± 1.9[b]	7.0 ± 0.9[b]	6.9 ± 1.1[b]	1.8 ± 0.3[b]
Cynomolgus	69 ± 5	29 ± 3	27 ± 2	5.3 ± 0.9	2.8 ± 0.5	2.4 ± 0.4	0.6 ± 0.1

Values are mean ± SEM; 8 monkeys per group.

[a]Significantly greater than CDCA ($p<0.01$, paired t-test).

[b]Significantly different from cynomolgus ($p<0.01$, Student's t-test).

keys, the pool size of CA was 3.5 times larger than CDCA, while the basal synthesis of both primary bile acids was equal. On the other hand, in cynomolgus monkeys both the pool size and basal synthesis of CA and CDCA were comparable (Table 2). The cebus monkey had twice the rate of total bile acid synthesis seen in the cynomolgus, even though the ratio of pool size to basal synthesis for CA was the same for both species. By contrast, the ratio of pool size to basal synthesis for CDCA was significantly smaller in cebus than cynomolgus (Table 2).

Basal Cholesterol Secretion

In keeping with BA secretion, the secretion rate for biliary cholesterol in cebus was 3 times that in cynomolgus monkeys (Table 2).

Incorporation of Injected Lipoprotein ^{14}C-Cholesterol into Biliary Sterols

The specific activities (SAs) of biliary cholesterol and total bile acids were determined for the total bile secreted during the 2-hr infusion of lipoprotein ^{14}C-cholesterol (Table 3). Irrespective of the lipoprotein infused, in both monkey species the SA of biliary cholesterol was significantly greater than that of total bile acids.

TABLE 3

Specific Activity of Biliary Cholesterol (BC) and Total Acids (BA) after LDL or HDL ^{14}C-Cholesterol Infusion of Infant Cebus and Cynomolgus Monkeys

Species and sterol	Infused		Average for both LPs
	LDL	HDL	
	(dpm·μmol^{-1})		
Cebus			
BC	2194 ± 66[a]	986 ± 218[a]	1504 ± 254[a]
BA	602 ± 102	206 ± 40	376 ± 88
Cynomolgus			
BC	2201 ± 700[a]	3443 ± 1082	2822 ± 641[a]
BA	502 ± 215	1662 ± 285	1082 ± 274

Values are mean ± SEM; 4 monkeys in each group.

[a]Significantly greater than BA ($p<0.01$, paired t-test).

When the SA of the primary bile acids, CA and CDCA, was calculated for the 2-hr infusion period, the SA of CA was always significantly greater than that for CDCA in both cebus and cynomolgus monkeys, again independent of the lipoprotein infused (Table 4).

TABLE 4

Specific Activity of Cholic (CA) and Chenodeoxycholic Acid (CDCA) after LDL or HDL ^{14}C-Cholesterol Infusion of Cebus and Cynomolgus Monkeys

Species and bile acid	Infused		Average for both LPs
	LDL	HDL	
	(dpm·μmol^{-1})		
Cebus			
CA	6834 ± 1024[a]	1706 ± 405[a]	3904 ± 1074[a]
CDCA	3173 ± 314	1013 ± 495	1939 ± 445
Cynomolgus			
CA	1961 ± 680[a]	3612 ± 1100[a]	2786 ± 675[a]
CDCA	1517 ± 504	2745 ± 815	2131 ± 501

Values are mean ± SEM of average SA during 2-hr infusion period; 4 monkeys per group.

[a]Significantly greater than CDCA ($P<0.02$, paired t-test).

Taurine/Glycine Conjugation

In the cynomolgus monkey, which conjugates up to 40% of its bile acid pool with glycine, the SAs of CA and CDCA conjugated with taurine or glycine were calculated following lipoprotein free ^{14}C-cholesterol infusion. The SA of glyco-CA was greater than that for tauro-CA, whereas the tauro-CDCA pool had a higher SA than glyco-CDCA (Table 5).

DISCUSSION

Because the SA of plasma LDL and HDL could not be controlled due to rapid exchange of label between these two lipoproteins, conclusions concerning the preferential incorporation of either LDL or HDL cholesterol by one species over the other could not be made. However, conclusions concerning the presence of distinct precursor pools for biliary cholesterol and primary bile acid conjugates within each species and within treatment groups remain valid. At least three inferences can be drawn from the experimental results.

First, in both species, regardless of the lipoprotein infused and assuming that constant infusion of labeled cholesterol for 2 hr had saturated the specific activity of the various cholesterol precursor pools equally, the injected lipoprotein free cholesterol was preferentially incorporated into biliary cholesterol rather than bile acids, and within the bile acid pool, into CA rather than CDCA. This is noteworthy because previous studies in rats (47-51), humans (52,53), and monkeys (54) have demonstrated that newly synthesized hepatic cholesterol is incorporated into bile acids in preference to biliary cholesterol. Other available evidence suggests that CA and CDCA have different precursor pools of cholesterol (e.g. lipoprotein vs de novo-synthesized), and that CDCA is preferentially derived from newly synthesized cholesterol (54-57). Our results would concur with these precursor-product relationships and further indicate that the injected lipoprotein cholesterol (injected as unesterified cholesterol) contributes more efficiently to the biosynthesis of CA than CDCA. We could not determine mass transfer from our data.

How the cholesterol pools are compartmentalized within the liver is not clear and was not addressed by this study, but the indication is that the two pools under discussion, i.e. cholesterol entering with lipoproteins and that synthesized de novo by hepatocytes, may be distinct (14,52). Further, their relative contribution to cholesterol catabolism and excretion as bile acids or biliary cholesterol differs qualitatively and quantitatively. An atypical pathway for chenodeoxycholic acid synthesis has been proposed by Mitropoulos and Myant (58), who suggest that the side chain of cholesterol is oxidized to 3β, 26-dihydroxy-cholest-5-ene before the formation of 7α-hydroxycholesterol, the presumed common precursor of cholic and chenodeoxycholic acids. Thus, it is possible that if the former reaction is quantitatively important in these monkeys, then cholic and chenodeoxycholic acids may not share the same biosynthetic pathways or precursor pool.

A second inference from the data would be that the two primary bile acids have different preferences for, or access to, taurine and glycine. We previously reported (27) that cebus and cynomolgus monkeys differ in their bile acid profiles with respect to their taurine and glycine conjugation, and that during taurine depletion in cynomolgus monkeys (capable of glycine conjugation) taurine was uniquely conserved in the CDCA pool. This and other studies (59-61) suggest that taurine may have preferred access to CDCA. From the SA data of cynomolgus in the current study it is clear that CA synthesized from injected lipoprotein free cholesterol was preferentially conjugated with glycine, and that CDCA preferred taurine for conjugation.

Finally, the equal basal synthesis rate and pool size for CA and CDCA in cynomolgus suggests a rather balanced turnover of the two primary bile acids in this Old World monkey. By contrast, in New World cebus the CDCA pool size was one-fourth that of CA and considerably smaller than the CDCA pool in cynomolgus. This was true even though the rate of basal synthesis for CDCA and CA were equal in cebus, and even though the rates for cebus were more than 2 times those in cynomolgus. This would indicate a uniquely rapid turnover of CDCA by the exclusively taurine-conjugating

TABLE 5

Specific Activity of Cholic (CA) and Chenodeoxycholic Acid (CDCA) Conjugated with Either Taurine or Glycine after Lipoprotein ^{14}C-Cholesterol Infusion of Cynomolgus Monkeys

Bile acid	Taurine (dpm·μmol^{-1})	Glycine (dpm·μmol^{-1})
CA	1625 ± 474[a,b]	5876 ± 1228[b]
CDCA	2306 ± 617[a]	1813 ± 321

Values are mean ± SEM. Each cell represents data from the same 8 monkeys.

[a]Significant difference between taurine and glycine conjugate of the same bile acid ($P<0.02$, paired t-test).

[b]Significant difference between CA and CDCA conjugated with the same amino acid ($P<0.02$, paired t-test).

cebus. By a similar token, addition of taurine to the isolated, perfused cat liver increased BA synthesis when taurine availability became limiting (62), and dietary taurine supplementation in guinea pigs induced a marked shift from glycine to taurine conjugation of BAs while substantially increasing CDCA synthesis and turnover (61). Since the CDCA pool may preferentially utilize newly-synthesized cholesterol (54-57), the greater taurine availability, greater CDCA synthesis and turnover and higher biliary cholesterol secretion rate in cebus further support the concept that significant differences in cholesterol metabolism (particularly hepatic cholesterol and BA synthesis) between cebus and cynomolgus underly their contrasting lipoprotein cholesterol distribution and metabolism.

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Phosphatidylinositol Metabolism during Fertilization in the Sea Urchin Egg

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ABSTRACT

Fertilization of the sea urchin egg results in a transient decline in the amount of phosphatidylinositol (PI) to a level equal to about 50% of that present in the unfertilized egg. This response begins as early as 15 seconds after insemination. The level of PI reaches a minimum at 30 seconds post-insemination, and returns to the original value between 2 and 5 min later. Pulse labelling studies with $^{32}PO_4$ and [^{3}H]-inositol showed that the incorporation of these two isotopes into 1-(3-sn-phosphatidyl)-L-myo-inositol 4,5-biphosphate [PtdIns(4,5)P_2] increased as much as 50% within one minute after insemination. This suggests that at least part of the reduction in PI levels represents the phosphorylation of PI to form PtdIns(4,5)P_2. We also found that the production of [^{3}H]-labelled 1D-myoinositol 1,4,5 triphosphate [Ins(1,4,5)P_3] present in the trichloroacetic acid (TCA) soluble fraction of eggs increased over five-fold during the first 10 min post insemination. The temporal correlation between the early burst of PtdIns(4,5)P_2 and Ins(1,4,5)P_3 formation and the transient increase in intracellular free calcium known to occur in the fertilized egg suggest that the production of PtdIns(4,5)P_2 and ultimately Ins(1,4,5)P_3 may be associated with calcium mobilization within the egg.
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INTRODUCTION

During fertilization the sperm binds to a specific receptor on the surface of the egg, and shortly thereafter the two cells fuse. This cell adhesion event or the membrane fusion process, or both, triggers a rapid, preprogrammed series of events which transforms the quiescent egg into a rapidly dividing zygote. This transformation (egg activation) is now known to involve changes in the rate of ion and nutrient transport (1,2), activation of NAD kinase (3), and a variety of protein kinases which are not yet fully characterized (4,5). Other changes include the elevation of cytoplasmic pH (6), increased protein and DNA synthesis (7,8) and the initiation of mitosis.

The earliest (and possibly the initiating) step in egg activation is a sperm-induced membrane depolarization. This initial depolarization triggers an action potential which is the first component of the activation potential (9). Concurrent with the later phase of the activation potential (45-180 sec) is a temporary elevation of intracellular free calcium within the egg. During this "calcium transient," the calcium concentration changes from undetectable levels to 2-5 μM, largely a result of calcium release from internal stores (10). The calcium transient is thought to serve as a major regulatory step in egg activation. It directly stimulates cortical vesicle exocytosis (thus preventing polyspermy) (11,12), initiates the non-mitochondrial burst of oxygen consumption (13,14) and indirectly results in increased tyrosine protein kinase activity (5). The calcium inophore A23187 has been used to parthenogenically activate many of the calcium stimulated responses to fertilization (14-16), and a combination of A23187 and NH_4Cl (to elevate cytoplasmic pH) has been used to achieve complete parthenogenetic activation of the egg with successful development to the pluteus stage (17).

The objective of the present study is to investigate the possibility that the calcium transient in the sea urchin egg is mediated by a burst of PI phosphorylation and subsequent hydrolysis to release inositol 1,4,5-triphosphate. Such a mechanism has been proposed in a variety of systems where calcium mobilization occurs in response to hormonal or neuronal stimulation (18-22). We have found that fertilization results in a rapid reduction in the amount of PI per egg beginning 30 sec after insemination, a concurrent increase in labelling of PtdIns(4,5)P_2 by either $^{32}PO_4$ or [^{3}H]-inositol, and increased labelling of Ins(1,4,5)P_3. Our data are consistent with the mechanism described above. The fact that these events correspond temporally with the calcium transient suggests that Ins(1,4,5)P_3 may be responsible for releasing calcium from internal stores as has been demonstrated in permeabilized hepatocytes (22).

MATERIALS AND METHODS

Sea urchins *(Lytechinus variegatus)* were collected in the Miami area and gametes were

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obtained by KCl injection. The eggs were then dejellied by washing them in seawater adjusted to pH 5.5 with HCl. The following chemicals were obtained from the indicated suppliers: [^{32}P] orthophosphate (carrier free), myo[2-^{3}H] inositol (10-20Ci/mmol) and [^{14}C] phosphatidylinositol (50mCi/mmol) Amersham Corp.; phospholipid standards, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), butylated hydroxytoluene (BHT), ethylenediamine tetraacetic acid (EDTA), Sigma Chemical Co.; Dowex Ag1 X8 (formate), BioRad.

Unfertilized eggs were suspended in millipore filtered sea water (FSW) as a 20% v/v suspension, and one group was fertilized by addition of 5 μl of "dry" (undiluted) sperm followed by gentle agitation. Typically, greater than 95% of the eggs exhibited fertilization membranes within 30-45 sec after sperm addition. After different periods of time, 100μl aliquots (200 mg protein) were removed, placed in 12 ml conical centrifuge tubes and immediately extracted with 4 ml of chloroform:methanol:HCl (2:1:0.0075, v/v/v) containing 50 μg/ml BHT. Then the pellet was re-extracted with 2 ml of the above. The extracts were combined, backwashed with 1.5 ml of 0.9% NaCl (wt/vol) in 0.01N HCl, and dried under N_2. The recovery of PI by this procedure was determined by including [^{14}C]-PI as an internal standard and was routinely over 90%. The recovery of the polyphosphoinositides was determined by parallel experiments using reagent grade PtdIns(4,5)P_2 and analysis by colorimetric phosphate assay and was found to be 84.8 ± 2.4%.

To quantitate PI, egg phospholipids (containing [^{14}C]-PI added prior to extraction as an internal standard) were analyzed by HPLC using a variation of a previously published technique (23). Chromatography was performed on a 250×4mm column of LiChrospher Si 100 II (5μm) (Merck) eluted with hexane:propanol: water (Fig. 1). Lipids were detected by absorbance at 205nm and quantitated by integration of the detector output. Fractions were collected and counted in a scintillation counter to determine the recovery of [^{14}C]-PI, which was 81.4% ± 3.5% through the extraction and chromatography steps. The polyphosphoinositides were analyzed by thin layer chromatography on Redicoat G (Supelco) plates (20×20cm × 0.5mm) developed first with chloroform:methanol: ammonia (4.3 M) (36:26:8) and then with n-propanol:ammonia (4.3 M) (6:4) in the same direction (24). Lipids were detected by iodine vapor ([^{3}H] and [^{14}C]-labelled) or by charring with sulfuric acid. The plates were divided into 0.5 by 2.5 cm regions which were scraped off and counted in a scintillation counter. Inositol phosphates were analyzed by the method of Berridge (25), and the eluates were freeze dried, resuspended in water, then freeze dried again to remove the formic acid before counting in a scintillation counter. Samples were corrected for quenching by the external standard ratio method. Inorganic phosphorus (26) and protein (27) were determined by colorimetric assays.

RESULTS

Effect of Fertilization on Egg Phosphatidylinositol Content

The phospholipid composition of eggs before, and at various times after fertilization was determined by HPLC as seen in Figure 1. Integration of the absorbance record revealed that PI accounted for 14.4 ± 4.0% of the phospholipids in the unfertilized egg. A similar value (13.2%) was obtained by phosphate analysis of the individual lipid classes in two of the experiments. Fertilization resulted in a rapid decline in the amount of PI per egg to 7.5 ± 4.3% (7.8% by phosphate analysis) within 30 sec (Fig. 2). This effect was often apparent as early as 15 sec post insemination. However, the values obtained at this early point were quite variable, possibly because of variations in the relative rate at which different populations of eggs were fertilized by the sperm. After 30 sec the PI levels

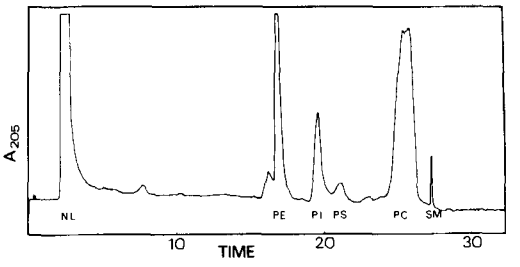

FIG. 1. HPLC analysis of unfertilized egg phospholipids. Lipid extracts containing 0.2 to 0.4 μmoles of lipid phosphate were injected in a volume of 50μl $HCCl_3$. The column was run at 35 C at a flow rate of 1ml per min. Solvent A consisted of hexane:2-propanol (3:4), and solvent B was water. The column was run isocratically with 1% v/v solvent B for 5 min, then a linear gradient of 1% to 8% B over 15 min, and finally held at 8% B for 25 min. The column was re-equilibrated by running a reverse gradient from 8% B to 1% B and run at 1% B for 90 min before injection of the following sample. This re-equilibration was necessary to obtain reproducible retention times. The separation and retention times obtained here were similar to those observed in an earlier publication (23). NL, neutral lipids; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin.

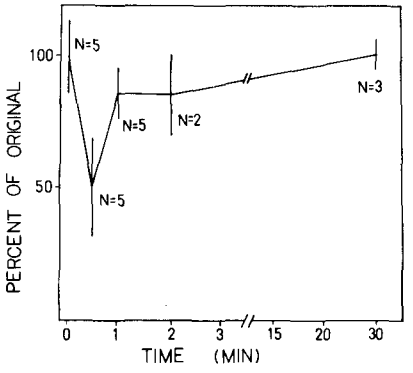

FIG. 2. Phosphatidylinositol content of sea urchin eggs before and at various points after fertilization. Aliquots from a 20% v/v suspension of unfertilized or fertilized eggs were incubated at 20 C for the indicated lengths of time. The lipids were then extracted and analyzed by HPLC as described in Materials and Methods. Phospholipids were quantitated by absorbance at 205nm, and the mole fraction of PI relative to the other phospholipids was calculated (for unfertilized eggs this was 14.4% by absorbance and 13.2% by phosphate analysis). Each sample was analyzed in duplicate, and the data are expressed as the mole fraction in a sample divided by the mole fraction in unfertilized eggs ± SD for (N) separate experiments. Sperm lipids accounted for less than 1.25% of the total in a given sample. Therefore, no correction was made for their presence.

quickly returned to the unfertilized value, and no further changes were detected.

Radiolabelling of Inositol Phospholipids

To determine if the above decline in PI content could be the result of increased production of the polyphosphoinositides, we prelabelled unfertilized eggs with $^{32}PO_4$, for 30 min, washed out the unincorporated isotope and divided the eggs into two groups. One group was fertilized, and both the unfertilized and fertilized eggs were incubated for various periods of time after which aliquots were removed and the lipids were extracted. The radiolabelled phospholipids were analyzed by a thin layer chromatography system which resolves both mono- and diphosphatidylinositol, radioautographed, and then quantitated by scintillation counting. The unfertilized eggs incorporated $^{32}PO_4$ primarily into PG, PA and PI with very little labelling of 1-(3-sn-phosphatidyl)-L-myo-inositol 4-phosphate [PtdIns(4)P] and PtdIns(4,5)P_2 (Figs. 3 and 4). However, within 30-60 sec after insemination, the incorporation into PtdIns(4)P and PtdIns (4,5)P_2 increased over 50% (Fig. 3 and Table 1). This suggests that fertilization results in either an increase in the levels of these polyphosphoinositides, an increase in their rate of turnover, or both.

In a similar series of experiments, we prelabelled unfertilized eggs with [^{3}H]-inositol, washed out the excess isotope, then fertilized one group and measured the incorporation into the phospholipids of unfertilized and fertilized eggs. As seen in Figure 4, the incorporation of [^{3}H]-inositol into PtdIns(4,5)P_2 increased in a manner similar to that observed in the $^{32}PO_4$ labelling experiment. Maximum incorporation occurred at 1 min post insemination and then gradually declined. A second rise in the incorporation of [^{3}H]-inositol occurred at 30 min after fertilization (Fig. 4). This later rise in PI metabolism originally was reported by Schmell and Lennarz (28). However, these investigators did not study the early responses to fertilization which we report on here.

Radiolabelling of Inositol Phosphates

To determine whether the production of PtdIns(4,5)P_2 was associated with the release of Ins(1,4,5)P_3, we prelabelled unfertilized eggs with [^{3}H]-inositol as above, then measured the incorporation into the inositol phosphates. As seen in Figure 5, the incorporation into Ins(4,5)P_2 and Ins(1,4,5)P_3 increased over five-fold after fertilization. Increased labelling of Ins(1,4,5)P_3 was first detectable at 1 min post insemination, reached a maximum at 10 min and then returned to the unfertilized levels. The labelling of inositol phosphate decreased by 70% immediately after fertilization, then slowly returned to the unfertilized level.

In summary, these results demonstrate that within 30-60 sec after fertilization, the amount of PI per cell begins to decline and the amount or turnover of PtdIns(4,5)P_2 increases. Between 1 and 2 min after fertilization, an increase in labelling of free Ins(1,4,5)P_3 becomes apparent possibly resulting from the hydrolysis of newly formed PtdIns(4,5)P_2.

DISCUSSION

The unfertilized egg is a resting cell which responds to sperm fusion with a rapid series of biochemical events which function to activate egg metabolism, prevent polyspermy and initiate development. The calcium transient is an early response to fertilization beginning 30 to 45 sec post insemination and lasting for 2 to 3 min (10). Morphological studies using the fluorescent probe aequorin have revealed that the release of intracellular calcium within the sea urchin egg begins at the point of sperm penetration and proceeds as a propagated wave through the ooplasm with a transit time of 6-9 sec (29). The

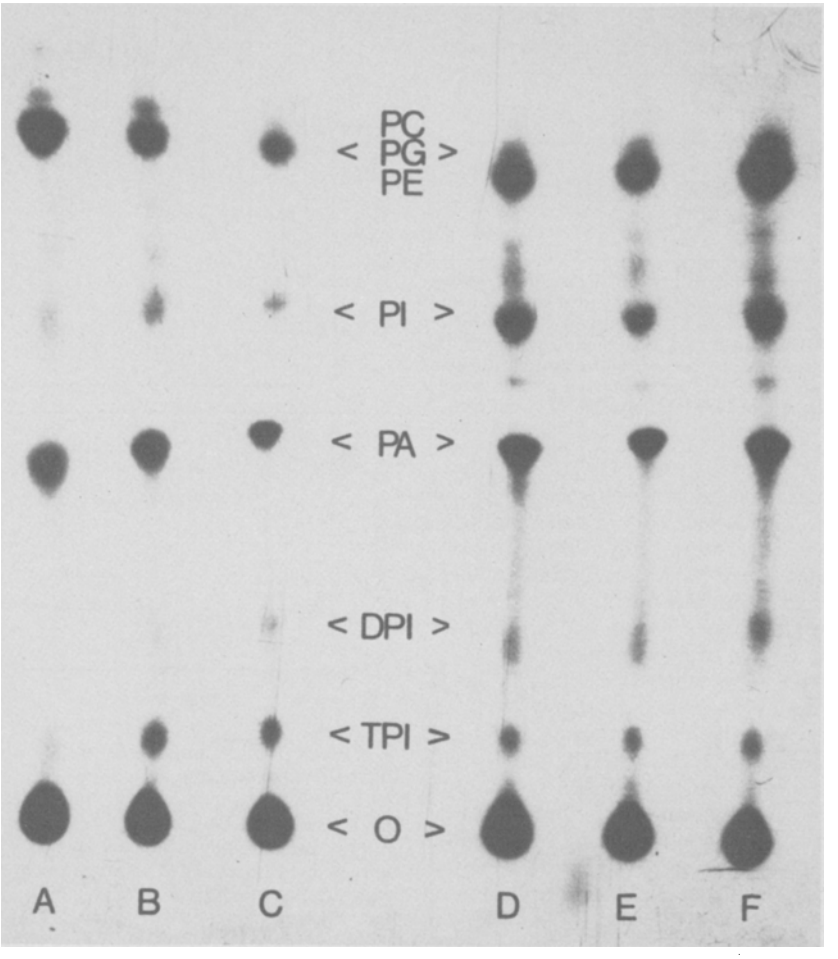

FIG. 3. Analysis of $^{32}PO_4$-labelled egg phospholipids by TLC and radioautography. Unfertilized eggs (20%, v/v) were prelabelled in FSW containing 1mCi/ml of ^{32}P-orthophosphate at 20 C for 30 min, then washed five times in 10 vol of FSW. The labelled eggs were divided into two groups, one of which was fertilized, and aliquots were removed for lipid analysis as described. The samples are: unfertilized (A), fertilized 30 sec (B), 1 min (C), 2 min (D), 5 min (E), 15 min (F). The center lane contained nonradioactive standards which are indicated by the following abbreviations: origin (O), PtdIns(4,5)P_2 (TPI), PtdIns(4)P (DPI), phosphatidiacid (PA), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and phosphatidylcholine (PC).

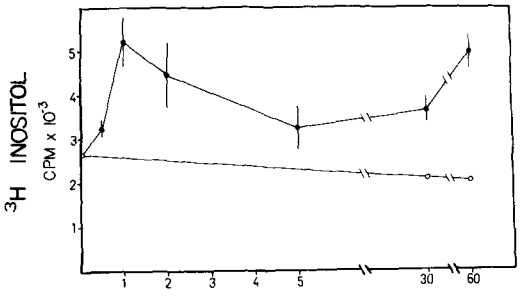

FIG. 4. ^{3}H-inositol labelling of PtdIns(4,5)P_2 during fertilization and early development. A 20% (v/v) suspension of unfertilized eggs was labelled with ^{3}H-inositol (125 μCi/ml) for 4 hr at 20 C, then washed five times in 10 vol of FSW. The eggs were divided into two groups, one of which was fertilized. Aliquots were removed from both groups, and the lipids were extracted and analyzed by thin layer chromatography. To determine the radioactivity in PtdIns(4,5)P_2, 0.5cm sections of the plate were scraped off and counted in a scintillation counter. The data have been corrected for a background radioactivity of 32 CPM per 0.5 cm region. Values are the average of duplicate samples of eggs, and the range is indicated by vertical bars. Unfertilized (○), fertilized (●).

TABLE 1

$^{32}PO_4$ Labelling of Egg Phospholipids at Fertilization

Time after fertilization	TPI	DPI	PI	PA
0 min	102 ± 18	42 ± 14	59 ± 18	232 ± 46
0.5 min	149 ± 29	85 ± 18	86 ± 21	255 ± 51
1 min	163 ± 22	88 ± 24	99 ± 24	378 ± 54
2 min	150 ± 27	74 ± 20	374 ± 53	440 ± 71

Eggs were radiolabelled and fertilized and the phospholipids extracted and analyzed by thin layer chromatography as in Figure 3. To quantitate incorporation into each phospholipid, 2.5 × 0.5 cm regions of the thin layer plate were scraped off and counted in a scintillation counter. The data are expressed as cpm/100 μg egg protein and represent the average of 4 analyses ± SD.

calcium transient is known to trigger the exocytosis of the cortical secretory vesicles and other events which help to establish a block to polyspermy (11,30). Although the calcium transient is a short-lived phenomenon, it triggers a series of long term processes which are required for successful initiation of development (17).

Despite the importance of calcium in fertilization and development, relatively little is known concerning the mechanism by which the calcium transient may be brought about. The turnover of phosphatidylinositol in response to hormone or neurotransmitter stimulation has been linked to calcium mobilization in a number of systems (reviewed in [31]). More recently, it has been realized that the phosphorylation of PI to form PtdIns(4,5)P_2 and its subsequent hydrolysis to produce diacylglycerol and Ins(1,4,5)P_2 is the more important step in releasing calcium from intracellular stores (22, 32,33). In the present study, we have sought to establish whether such a pathway occurs in the sea urchin egg in response to fertilization. A previous study established that PI turnover increased in response to fertilization. However, this study did not address the early responses to fertilization (less than 5 min post insemination) which might participate in the calcium transient (28). We have found that within 30 sec after insemination, the amount of PI in the egg declines by about 50%, then quickly returns to its original value. A similar phenomenon has been observed in platelets where about 33% of the cellular PI is hydrolyzed within 30 sec after addition of thrombin (23,34). Such a decline in PI content could represent hydrolysis of PI to form lysophosphatidylinositol as has been shown to occur during the stimulation of prostaglandin synthesis in 3T3 cells and kidney slices by bradykinin or angiotensin II (35,36). That such a mechanism may occur during fertilization

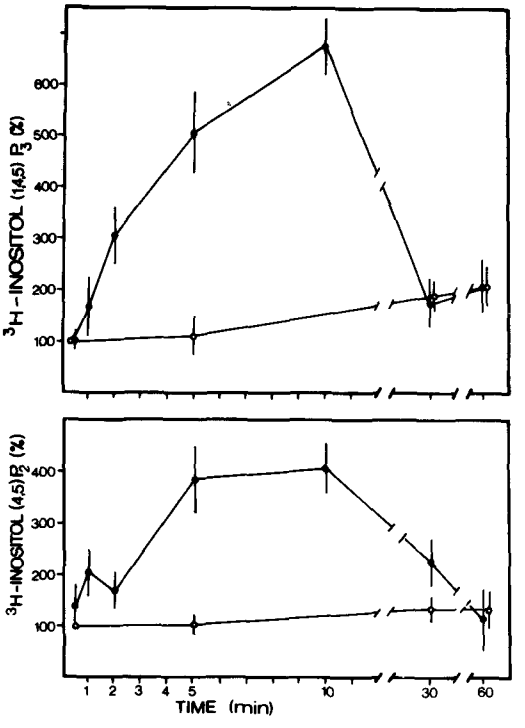

FIG. 5. Production of inositol phosphates by eggs during fertilization and early embryonic development. A 20% (v/v) suspension of unfertilized eggs was labelled with ^{3}H-inositol (125 μCi/ml) as in Figure 4, except that the incubation period was 6 hr. The eggs were then washed and divided into two groups, one of which was fertilized. 250 μl aliquots (0.5 mg egg protein) were removed at the indicated times, and the eggs were pelleted by gentle centrifugation. The proteins were precipitated with 250 μl of 30% (wt/vol) TCA and centrifuged. The supernate was removed and the pellet washed with a second 250 μl aliquot of 15% TCA. The supernates were pooled and extracted five times with 5ml of ethyl ether, then neutralized with 1N NaOH. The different inositol phosphates were analyzed by ion exchange chromatography (25). The data are the average of two experiments (range indicated by vertical bars), and the incorporation of ^{3}H-inositol is expressed relative to that observed in the unfertilized eggs at 30 sec in order to correct for differences in inositol incorporation between experiments. For purposes of comparison, the average incorporation of ^{3}H-inositol/mg unfertilized egg protein was 2,326 cpm for Ins P (not shown), 177 cpm for Ins(4,5)P_2 (bottom), and 163 cpm for Ins(1,4,5)P_3 (top). Unfertilized eggs (○), fertilized eggs (●).

has been suggested by a recent report implicating a phospholipase A_2 in the mechanism of cortical vesicle exocytosis (37). We therefore consider it possible that some of the loss of PI at fertilization may reflect the action of a phospholipase A_2. However, we were unable to

detect the production of free arachidonic acid in eggs prelabelled with [^{14}C]-arachidonate-PI and then fertilized (data not shown). One possible explanation for this is that free arachidonic acid could be oxidized rapidly through the lipoxygenase and cyclooxygenase pathways which are active after fertilization (38) and appear to play a role in preventing polyspermy (39,40).

Another mechanism which would cause a reduction of PI is the phosphorylation of PI to form PtdIns(4,5)P_2 and its subsequent hydrolysis producing Ins(1,4,5)P_3, as mentioned above. This mechanism has been shown to occur during the stimulation of secretory cells in the liver, pancreas and salivary gland (20,21,32). More recently, fertilization of *S. purpuratus* eggs was found to result in a 30-50% increase in the levels of PtdIns(4)P and PtdIns(4,5)P_2 within 30 to 60 sec after fertilization. This finding suggests that the phosphorylation of phosphatidylinositol is an early response to fertilization (41). Our results confirm and extend this finding. In the present study, fertilization resulted in an increase in the labelling of PtdIns(4,5)P_2 as measured by both $^{32}PO_4$ and [^{3}H]-inositol incorporation beginning as early as 30 sec post-insemination, which corresponds temporally with the initiation of the calcium transient. Since the eggs were prelabelled before fertilization and then washed, the changes in isotope incorporation do not reflect increased transport but indicate either an increase in the amount or turnover of PtdIns(4,5)P_2. The increased labelling seen here seems inconsistent with the agonist-stimulated decline of radiolabelled PI, PtdIns(4)P, and PtdIns(4,5)P_2 observed in the fly salivary gland (32). However, the unfertilized sea urchin egg is very inactive metabolically, and our short term labelling conditions did not come close to equilibrium labelling of the phospholipids. We therefore would not expect to observe the initial hydrolysis of the existing (largely unlabelled) phospholipids, but we would expect to see the incorporation of the highly radioactive precursor pools into these phospholipids as they begin to turn over. By two to five min after fertilization of the labelling of PtdIns(4,5)P_2 returned to a level close to that of the unfertilized egg, suggesting that the rate of turnover had decreased.

If the fertilization stimulated increase in polyphosphoinositide metabolism plays any role in calcium mobilization, we would expect that the level of Ins(1,4,5)P_3 would increase coincident with the stimulation of PI turnover. We observed that the incorporation of [^{3}H]-inositol into Ins(4,5)P_2 and Ins(1,4,5)P_3 increased over five-fold during the first 10 min after fertilization. The initial increase in inositol di- and triphosphate labelling is not apparent until 30-60 sec and thus follows the increased turnover of the phosphoinositides and the onset of the calcium transient (29). Instead of preceding the calcium transient, the rise in [^{3}H]-inositol phosphate labelling seems to more closely correspond to the occurrence of the cortical reaction which begins as intracellular calcium levels reach a maximum (29). Additionally, inositol phosphate labelling continued to increase for 10 min, during which time the labelling of PtdIns(4,5)P_2 is declining. This suggests that inositol (1,4,5)P_3 is removed from the phospholipid and accumulates in the egg although our data does not rule out other mechanisms. The observation that Ins(1,4,5)P_3 production remains high for 10 min post insemination would lead us to predict that the levels of intracellular free calcium would remain high for the same period. However, it is known that the calcium transient lasts for only 2 to 3 min (10). The probable explanation for this discrepancy lies in the fact that the egg begins to pump calcium out of the cell actively after fertilization, removing up to 30% of the total intracellular calcium with a halftime of 9-14 min (42). Thus free calcium would accumulate in the cytoplasm only as long as the rate of calcium release is greater than the rate at which it is pumped out or sequestered into intracellular compartments.

The data now available seem to indicate that the following sequence of events occurs after insemination. First is the rapid conversion of PI into di- and triphosphoinositides, which is easily detectable by 30 sec and even 15 sec in *S. purpuratus* (40). Approximately 50% of the cellular PI is consumed during this period, although some of this loss may be due to other mechanisms. Next, the calcium transient which begins between 20 and 30 sec, reaches a maximum by 45 sec and persists for 2-3 min. By 60 sec the chemical levels of PI have been largely restored, and radiolabelled Ins(1,4)P_2 and Ins(1,4,5)P_3 begin to accumulate in the cytoplasm. Whether significant amounts of inositol phosphates are produced prior to this is not known because we can measure only the accumulation of the material produced from tritium labelled precursors. This occurs only when the rate of synthesis exceeds the rate of breakdown. Since the PtdIns(4,5)P_2 precursor is available as early as 15-30 sec post insemination, it is possible that inositol di- and triphosphate could be produced transiently during the initial phase of the calcium transient. However, the accumulation of inositol di- and triphosphate seen here is more likely to function in maintaining elevated

calcium levels and bringing about the cortical reaction.

The results presented here are concerned primarily with the mechanism for the release of intracellular calcium during the response of the egg to fertilization. The recent demonstration that injection of Ins(1,4,5)P_3 into unfertilized eggs induced the cortical reaction and other aspects of egg activation (43) suggests that the release of Ins(1,4,5)P_3 within the egg would indeed have a biological effect. However, it is also possible that the production and hydrolysis of polyphosphoinositides may have other biological implications. For example, the action of a phospholipase C to release Ins(1,4,5)P_3 also would produce diacylglycerol (31). This not only represents a physical change in the polarity of the membrane lipids, but also may directly affect the activity of membrane associated enzymes such as protein kinase C, which is stimulated by diacylglycerol (33,34). The activation of protein kinase C or other similar enzymes would provide a mechanism by which the changes in phosphoinositide metabolism could initiate long term metabolic changes which participate in development of the zygote.

ACKNOWLEDGMENTS

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Differential Effects of Lipoxygenase Products on FMLP and LTB_4 Evoked Neutrophil Aggregation

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ABSTRACT

There is evidence that the endogenous biosynthesis of LTB_4 is involved in the aggregation of human neutrophils induced by the chemotactic peptide f-met-leu-phe (FMLP). If LTB_4 mediates this aggregatory response, then agents which desensitize neutrophils to LTB_4 should inhibit the cellular response to FMLP. Since many lipoxygenase products modulate other neutrophil responses to LTB_4 and FMLP, we have investigated the effects of lipoxygenase products on LTB_4- and FMLP-initiated aggregation.

Prior exposure to low concentrations of LTB_4 (0.5-10nM) inhibited subsequent aggregation to the same agent (50nM), but it did not influence the response to FMLP (10^{-7}M). Relatively high concentrations of 5-HETE (5-50μM) inhibited aggregation initiated by either stimulus. Although the hydroperoxy derivative 5-HPETE also inhibited the response to LTB_4, in the relatively narrow concentration range of 1-4μM it stimulated FMLP-induced aggregation. This latter effect was confirmed using 12 cell preparations from six separate donors; it (the activity of 5-HPETE) was not mimicked by other 5-lipoxygenase products, including LTB_4, nor the dihydroperoxide 8,15-DiHPETE. Our results indicate that neutrophil aggregation in response to LTB_4 or FMLP can be selectively potentiated or inhibited. On the basis of these data we conclude that the endogenous synthesis of LTB_4 is not directly involved in the neutrophil aggregatory response to FMLP, although the hydroperoxy intermediate 5-HPETE may act to enhance the cellular response.
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INTRODUCTION

Exposure of human neutrophils to the chematic peptide f-met-leu-phe (FMLP) initiates the biosynthesis of leukotriene B_4 (LTB_4) (1). Because LTB_4 is a potent stimulus for a number of neutrophil responses (2), it is of interest to determine whether FMLP-evoked processes are mediated by LTB_4. In this regard, O'Flaherty et al have reported that neutrophil aggregatory responses elicted by certain agents, including LTB_4 and FMLP, are inhibited by prior exposure of the cells to low levels of LTB_4 (3). These authors suggested that the neutrophils were desensitized to LTB_4 by means of this prior exposure, and that the response to a subsequent stimulus whose effect is mediated by LTB_4 would be inhibited. Therefore, they concluded that the FMLP-initiated aggregatory response is a consequence of endogenous biosynthesis of LTB_4. Support for this hypothesis includes studies in which inhibitors of lipoxygenase were shown to inhibit neutrophil aggregation (4). Other investigations, however, have indicated that FMLP is unable to stimulate the release of arachidonate from phospholipid stores and is, thus, able to initiate LTB_4 production only if free arachidonic acid is already available (5).

A variety of studies has shown that lipoxygenase products may either potentiate or inhibit responses to subsequent stimuli such as chemotactic peptides, platelet activating factor (PAF) or leukotriene B_4. For example, 5-HETE and 5-HPETE enhance the degranulation response of neutrophils to PAF or LTB_4 (6.7). In addition, we recently have demonstrated that LTB_4 is a potent enhancer of the production of superoxide anions induced by FMLP (8). LTB_4 is thus capable of functioning both as a primary stimulus and as a modifying agent for neutrophil responses.

In the present study, we have compared the effects of LTB_4 and a variety of other lipoxygenase products on LTB_4-evoked aggregation with the effects of these agents on aggregation initiated by FMLP. If the aggregatory response to FMLP is solely a consequence of LTB_4 synthesis, then the lipoxygenase products should exhibit qualitatively similar effects on the two stimuli.

METHODS

Bovine serum albumin (BSA), FMLP and cytochalasin B were purchased from Sigma Chemical Company, St. Louis, Missouri. Hank's balanced salt solution (HBSS) was obtained from the Grand Island Biological Company, Grand Island, New York.

Leukotriene B_4 (LTB_4) and 5(S), 12(S)-DiHETE were donated by J. Rokach, Merck Frosst Canada, Inc., Quebec, Canada.

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5(R,S)-HETE was synthesized from arachidonic acid via 5-HETE-δ-lactone by methods previously described; it was identified by UV, NMR and GC MS criteria (9). 5(R,S)-HPETE was produced via autoxidation of arachidonic acid in the presence of vitamin E (10). Vitamin E controls the autoxidation and allows formation of equal amounts of six possible HPETE's in high yield (10). The mixture was dissolved in chloroform, injected onto a μ porasil HPLC straight phase column and eluted with hexane: isopropanol:glacial acetic acid (98.4/1.6/0.1, v/v/v) at a flow rate of 3 ml/min and monitored via detection at 235 nm. The identification of 5-HPETE as the sixth and final product to elute was verified by chemical reduction followed by injection onto and elution from a reverse phase HPLC column with retention time identical to authentic 5-HETE. 12(S)-HETE was synthesized biologically from porcine platelets and isolated by reverse phase HPLC.

8(S), 15(S)-dihydroperoxy-5, 8, 11-*cis*-9, 13-*trans*-eicosatetraenoic acid (8,15-DiHPETE) was prepared from arachidonic acid by incubation with soybean lipoxygenase (type IV, Sigma). Arachidonic acid (Supelco Co., Bellefonte, Pennsylvania) was converted to the sodium salt and incubated for one hr in 60 ml of .05N sodium borate buffer, pH 8.7 with soybean lipoxygenase (3.2mg protein). 8,15-DiHPETE was isolated by reverse phase chromatography monitored by U.V. detection at 280 nm.

Leukotriene A_4, methyl ester was obtained as a gift from the Upjohn Company. The stock solution was dissolved in diethylether:triethylamine:n-hexane (15/1/84) and stored at -78 C. Aliquots of the stock were converted to the lithium salt prior to its use by methods described (11).

The lithium salt was stored at -78 C and used within 5 days of preparation. LTA_4 was dissolved in BSA solution (50mg/ml) immediately prior to addition to cells. Such concentrations markedly stabilize LTA_4, extending its half life from seconds to several minutes (11).

Neutrophil Suspensions

Human neutrophils were isolated from heparinized venous blood from healthy adult volunteers. Isolation was initiated by enhanced erythrocyte sedimentation followed by layering of plasma and buffy coats onto ficoll-Hypaque gradients. The gradients were lysed with 0.15N NH_4Cl. The resulting neutrophils were washed and resuspended in HBSS. This isolation procedure consistently yielded neutrophils with greater than 98% purity and viability.

Neutrophil Aggregometry

Neutrophil aggregation was measured via a Payton aggregometer (12). The recorder was calibrated at maximum light transmittance using HBSS only. Heights of aggregatory waves were measured and recorded in mm.

RESULTS

Selected lipoxygenase products were found to inhibit or enhance the neutrophil aggregatory responses to LTB_4 and FMLP. It also was observed that the response to one primary stimulus could be modulated independently of the other. In testing LTB_4 itself as a modulating agent, we made use of the fact that cells show little reaction in the absence of cytochalasin B (13). Under these conditions, preincubation of neutrophils with LTB_4 greatly inhibited subsequent aggregatory responses initiated by a second exposure to LTB_4 plus cytochalasin B (Fig. 1). This is the result expected from previous studies (3). In contrast, prior exposure to LTB_4 had no effect on FMLP-induced aggregation (Fig. 1). The selective modulating action of LTB_4 was not mimicked by the 5,12-dihydroxy analog, 5(S), 12(S)-DiHETE; in concentrations from 10nM to 5μM this double lipoxygenase product

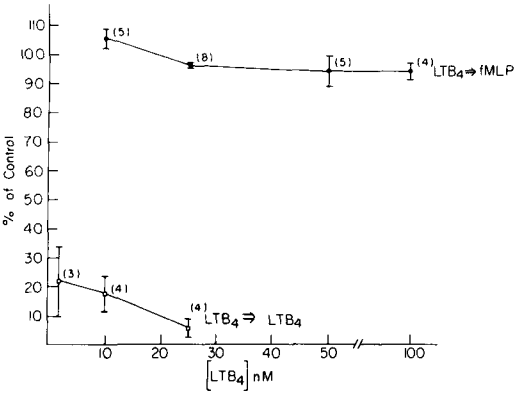

FIG. 1. Effect of leukotriene B_4 on neutrophil aggregations induced by FMLP on LTB_4. Human neutrophils (0.5ml, 1.0×10^7 cells 1ml) were exposed to LTB_4 (or solvent) for 5 min at 37 C in a dual chamber aggregometer prior to initiation of aggregatory responses by (•) FMLP (10^{-7} M) or (□) LTB_4 (50nM, following equilibration with cytocholasin B [1μg/ml]). The heights of aggregatory waves were measured from the stable baseline to the plateau (achieved in 5 min) resulting from the addition of aggregatory stimulus. The 100% value is obtained in the absence of lipid modifier. The sample number is given in parentheses, and brackets denote the SEM.

was completely inactive. Very high concentrations of the platelet lipoxygenase product 12-HETE (10-25μM) did weakly mimic the modulating activity of LTB_4 (Fig. 2).

Exposure of neutrophils to 5-HETE (1-50μM) resulted in a dose-dependent inhibition of both FMLP- and LTB_4-evoked aggregation (Fig. 3). Aggregation to LTB_4 also was inhibited by 5-HPETE. However, the effect of 5-HPETE on the FMLP response was biphasic. Potentiation was observed in the 1-4μM concentration range of 5-HPETE. Higher concentrations were inhibitory (Fig. 4). This biphasic action was not

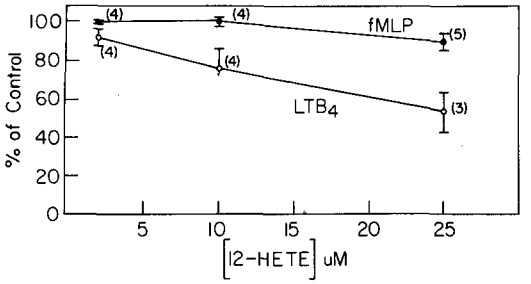

FIG. 2. Effect of 12-HETE on neutrophil aggregatory response to (•) FMLP or (○) LTB_4. Human neutrophils were incubated in a dual chamber Payton aggregometer as described in Figure 1 with the indicated concentrations of 12-HETE (or solvent) for 5 min. Aggregations were then initiated by exposure of the cells to (•) FMLP (10^{-7} M) or (○) LTB_4 (50nM, following equilibration with CB [1μg/ml]).

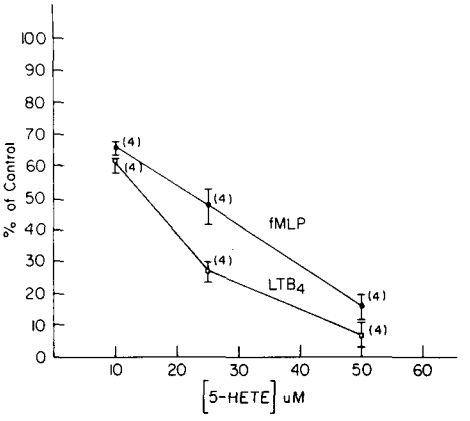

FIG. 3. Inhibition of FMLP or LTB_4-induced aggregation by 5-HETE. Human neutrophils (0.5ml, 1.0×10^{-7} cells/ml) were incubated at 37 C in a dual chamber Payton aggregometer. Incubations in the presence of the indicated concentration of 5-HETE (or solvent) were performed for 5 min prior to addition of the following aggregatory stimuli (•) FMLP (10^{-7}M) or (□) LTB_4 (50nM, following equilibration with CB).

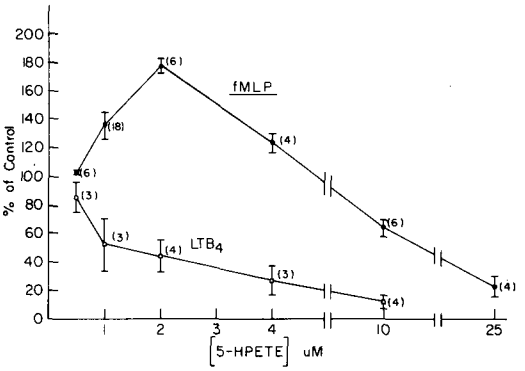

FIG. 4. Effects of 5-HPETE on neutrophil aggregatory responses to FMLP or LTB_4. Human neutrophils were incubated in a dual chamber Payton aggregometer as described in Figure 1 with the indicated concentrations of 5-HPETE (or solvent) for 5 min. Aggregations were then initiated by exposure of the cells to (•) FMLP (10^{-7}M) or (□) LTB_4 (50nM, following equilibration with CB [1μg/ml]).

simply dependent on the hydroperoxide moiety in 5-HPETE; no potentiation was observed using the dihydroperoxide 8(S), 15(S)-DiHPETE in the same concentrations. In leukocytes 5-HPETE is metabolized to the leukotriene epoxide LTA_4, and exogenous 5-HPETE is converted to LTA_4 in low yield (14). We added LTA_4 as a potential modulating agent in concentrations from 10-200nM, and it also exhibited no effect on FMLP-induced aggregation.

DISCUSSION

The present results show that selected lipoxygenase products can exhibit a different effect on LTB_4- and FMLP-initiated neutrophil aggregation. Exposure of neutrophils to LTB_4 'desensitized' the aggregatory response of the cells to LTB_4 but did not influence the FMLP aggregatory response. Thus, it appears that the systems for LTB_4- and FMLP-induced aggregation are not identical. This conclusion was further supported by the results obtained using 1-4μM 5-HPETE as the FMLP aggregatory response was enhanced, while LTB_4 aggregations were desensitized. The enhancement of FMLP evoked aggregations by this narrow range of concentrations of 5-HPETE was seen repeatedly using 12 different cell preparations from six donors. Enhancement of FMLP-induced aggregation was not exhibited by 5-HPETE and was not dependent solely on the hydroperoxy functional group of 5-HPETE as 8,15-DiHPETE was without effect. Although endogenously synthesized lipoxygenase products may facilitate the potentiation exhibited by 5-HPETE, neither

LTB_4 nor the 5,6 epoxide of LTA_4 act in this manner.

The lack of effect of LTB_4 on FMLP-evoked aggregatory responses was in contrast to the finding of O'Flaherty et al (3), who observed a strong inhibition by LTB_4 of the FMLP aggregatory response. In the latter studies, measurements were made by a Coulter counter method which detects changes in the number of large particles (two or more cells in aggregation). It is possible that LTB_4 exerts subtle influences on FMLP-evoked increases in large particle percentage which are not detected via the Payton aggregometer. O'Flaherty et al (3) suggested that the inhibition of FMLP-initiated aggregation by prior exposure to LTB_4 resulted because the FMLP aggregatory response is mediated by LTB_4. However, the results of our more extensive study of the two aggregatory stimuli indicate that LTB_4 biosynthesis is distinct from the FMLP aggregatory response.

Both 5-HETE and high levels of 5-HPETE inhibited FMLP and LTB_4-evoked aggregation while, as noted above, low levels of 5-HPETE enhance the FMLP response. Although these compounds generally are regarded as a precursor (5-HPETE) or a byproduct (5-HETE) of leukotriene biosynthesis, there have been suggestions that they regulate biological activities independent of the leukotrienes. Goetzl and co-workers reported 5-HETE to exhibit chemotactic activity (15) and to reverse the inhibition of migration achieved by preincubating neutrophils in 5,8,11,14-eicosatetraenoic acid (16). 5-HPETE subsequently was determined to exhibit chemotactic activity and to act as a chemotactic deactivator for a variety of homologous and nonhomologous stimuli (17). Similarly, O'Flaherty et al have noted that 5-HPETE and 5-HETE are able to modulate the neutrophil degranulation response to LTB_4 and PAF (6,7). We recently have reported that 5-HPETE and, to a lesser extent, 5-HETE enhance to oxidative response of human neutrophils to FMLP (18). Potential mechanisms for 5-HPETE-induced responses include covalent binding of the reactive hydroperoxide moiety to protein (as described in model systems by Shimasaki et al [19]). Esterification of 5-HETE into membrane phospholipids has been reported (20,21) and offers other possibilities.

In conclusion, evidence indicates that the FMLP-evoked aggregatory response is not a consequence of LTB_4 biosynthesis. The enhancement of FMLP-initiated aggregation by moderate levels of 5-HPETE suggests that other lipoxygenase products or perhaps 5-HPETE itself may be a component of the FMLP aggregatory response.

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Elongation Systems Involved in the Biosynthesis of Erucic Acid from Oleic Acid in Developing *Brassica juncea* Seeds

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ABSTRACT

In the presence of NADPH and malonyl-CoA, the cell-free extract of developing *Brassica juncea* seeds catalyzes the elongation of ^{14}C-oleoyl-CoA to radioactive C20:1 and C22:1. The elongation of C18:1 to C20:1 shows no marked preference for NADPH or NADH. On the other hand, the elongation of C20:1 to C22:1 exhibits a pronounced preference for NADPH. Moreover, the latter elongation system (C20:1 → C22:1) is more sensitive to inhibition by trichloroacetate and sodium metabisulfite. Inclusion of polyvinylpolypyrrolidone in the grinding buffer for preparation of the cell-free extract enhances the elongation activity by 5-6 fold. ^{14}C-Stearoyl-CoA, but not ^{14}C-palmitoyl-CoA, also is elongated by this system. The results presented here suggest certain degrees of dissimilarity between the first (C18:1 → C20:1) and second elongation (C20:1 → C22:1) systems.
Lipids 20:361-366, 1985.

INTRODUCTION

Erucic acid is associated primarily with the seeds of genus *Brassica* (1,2) genus *Sinapsis* (1), *Crambe abyssinica* (3), *Tropaeolum majus* (4), *Limnanthes alba* (5) and *Simmondsia chinensis* (Link) (6). In vivo experiments with these seeds have established that the biosynthesis of C20:1 and C22:1 fatty acids occurs exclusively by chain elongation of preformed oleate and not by a complete de novo process (7-14); this conclusion has been supported by in vitro studies (8,10,12,15).

In this paper we describe the preparation and properties of a cell-free extract from developing *Brassica juncea* seeds which catalyzes the elongation of [1-^{14}C]oleoyl-CoA to C20:1 (11c) and C22:1 (13c). The elongation of C18:1 to C20:1 and of C20:1 to C22:1 may involve different isozymes based on differential inhibition and reductant requirements of the two elongation steps.

MATERIALS AND METHODS

Plant Material

B. juncea plants (Bulk 78, developed by P.E. Knowles of the University of California, Davis) were harvested from fields on May 26, 1983. Pods within 6-9 in. of the flowering head were discarded, because of the low content of erucic acid (< 10%) in their seeds. Seeds collected for biochemical studies were green, and their erucic acid content was 34% of the total fatty acids. Pods were stored at -20 C.

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Substrates and Reagents

[2-^{14}C]Malonyl-CoA (59 Ci/mol), [1-^{14}C] oleoyl-CoA (56.8 Ci/mol), [1-^{14}C]stearoyl-CoA (58 Ci/mol) and [1-^{14}C]palmityol-CoA (57 Ci/mol) were purchased from Amersham Searle. Acyl-CoA esters, fatty acids, polyvinylpolypyrrolidone (PVPP) and other biochemicals were purchased from Sigma Chemical Company. *cis*-11 [1-^{14}C-Eicosenoyl-CoA (3.01 Ci/mol) prepared by M. Pollard (15) was purified by reverse-phase ion-pair high performance liquid chromatography (16). Hydrazine hydrate and cyclopentene were from Aldrich. Acyl carrier protein (ACP) from *Escherichia coli* was purchased from CalBiochem.

Preparation of Cell-Free Homogenate

Fresh or frozen (-20 C) *B. juncea* seeds (1 g) were ground with 10 ml of 0.08 M HEPES buffer, pH 7.2, containing 0.32 M sucrose and 10 mM mercaptoethanol (buffer A) and 1.5 g of PVPP and gently squeezed through two layers of Mira cloth to obtain cell-free homogenate as filtrate (5-6 ml, 4-5 mg/ml).

Measurement of Elongation Activity in Subcellular Fractions

Fresh *B. juncea* seeds (2 g) were crushed gently in a mortar with 20 ml of buffer A and 1.5 g of PVPP and gently squeezed through two layers of cheesecloth. The resulting homogenate was centrifuged in a swinging bucket rotor at 10,000 xg for 30 min. The floating fat pad was collected and washed twice by resuspending in buffer A and recentrifuging. The original sediment and supernatant obtained after collecting the fat pad were mixed gently and centrifuged

at 100 xg for 20 min. The supernatant subsequently was centrifuged at 2,000 xg for 20 min, then at 15,000 xg for 20 min and finally at 100,000 xg for 60 min to obtain 2,000 xg, 15,000 xg and 100,000 xg pellets. These pellets were resuspended in buffer A and centrifuged for 20 min at 100 xg (for 2,000 xg pellet), at 2,000 xg (for 15,000 xg pellet) and at 15,000 xg (for 100,000 xg pellet). The supernatants were centrifuged respectively at 2,000 xg, 15,000 xg and 100,000 xg to obtain the pellets. This step was useful in removing most of the chlorophyll from 15,000 xg and 100,000 xg pellets. Pellets were suspended in 1 ml of 0.08 M HEPES, pH 7.2, containing 10 mM mercaptoethanol, and 0.05 ml aliquots were assayed for elongation activity.

Assay for Elongation Activity

Unless otherwise indicated, 50 μl (about 200 μg protein) of the cell-free homogenate was incubated for 1 hr at 30 C with 17 μM [1-^{14}C] oleoyl-CoA, 1 mM malonyl-CoA, 0.5 mM NADPH, 1 mM ATP, 2 mM $MgCl_2$, 1 mM CoASH and 0.08 M HEPES pH 7.2 buffer in a total volume of 0.1 ml. The reaction mixture was saponified with 10% KOH and acidified with 5M H_2SO_4. The resulting fatty acids were extracted with petroleum ether and analyzed by radio-GLC as methyl esters (17) or by HPLC as isopropylidine hydrazides (18).

For analysis of lipid classes, the incubation was terminated by adding 0.1 ml acetic acid. Lipids were extracted using hexane-isopropanol (3:2, v/v) (18) and analyzed by TLC (19). Argentation TLC was done as described elsewhere (17). Reductive ozonolysis of unsaturated fatty acid methyl esters was done according to a microscale modification of the method of Steim and Nicolaides (20). Radioactive aldehyde-ester fragments were analyzed by radio-GLC.

Chlorophyll determination was done according to a method published before (21). Protein was assayed by a dye-binding method (22).

RESULTS

In vitro experiments demonstrating the elongation of [1-^{14}C]oleoyl-CoA to C20:1 and C22:1 in cell-free extracts of developing *B. juncea* seeds have been reported earlier (8); the present investigation is an extension of the previous studies.

In an effort to enhance the elongation activity, the inclusion of polyvinylpolypyrrolidone (1.5 g/g seeds) in the grinding buffer increased the elongation activity by almost 5-6 fold (from 8.0 nmoles/hr/g seed without to 57 nmoles with PVP). Other commonly used compounds for protection of plant enzymes, such as ascorbate and sodium metabisulfite, did not provide any further increase in elongation activity. On the contrary, the latter inhibited elongation. Inhibition by sodium metabisulfite ($Na_2S_2O_5$) will be discussed later.

When a cell-free homogenate was incubated with [1-^{14}C]oleoyl-CoA, malonyl-CoA, and NADPH, elongation of C18:1 to C20:1 and C22:1 occurred (Fig. 1). Argentation TLC and reductive ozonolysis showed that C20:1 and C22:1 were formed by successive additions of C_2 units to C18:1.

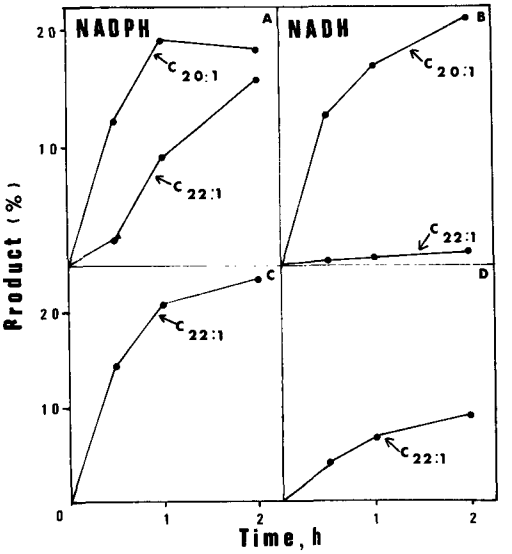

FIG. 1. Effect of reduced pyridine nucleotides on elongation of C18:1 (A and B) and C20:1 (C and D) CoAs. Experimental procedures are described under Table 4.

Cofactor Requirements for Elongation of C18:1-CoA

The cofactor requirements for elongation of [1-^{14}C]oleoyl-CoA to C20:1 to C22:1 by the cell-free extract of developing *B. juncea* seeds are given in Table 1. Malonyl-CoA, and not acetyl-CoA, served as the elongating unit. ATP was required for maximum elongation activity; in its absence, formation of C20:1 was markedly decreased without any significant change in the level of C22:1. Addition of ACP or CoASH did not have any effect on elongation. However, in the absence of both ATP and CoASH, a considerable drop in both C20:1 and C22:1 was noted. In the absence of reduced pyridine nucleotides, elongation activity did not occur. In the presence of NADPH alone, both C20:1 and C22:1 were formed, whereas in the presence of NADH,

TABLE 1

Effect of Various Cofactors on Elongation of [1-^{14}C] Oleoyl-CoA by Cell-Free Extract of *Brassica juncea* Oilseeds

	Product form (nmoles)/hr/ml extract	
	C20:1	C22:1
Control	12.9	5.2
NADPH (1 mM) - NADH	12.8	5.8
NADH (1 mM) - NADPH	11.8	trace
- NADPH - NADH	0.7	0.0
- ATP	6.0	4.9
- CoASH	13.3	5.1
- ATP - CoASH	5.4	2.2
+ ACP (100 μM)	12.6	4.7

For control, 0.1 ml extract (420 μg protein) was incubated for 30 min at 30 C with 17 μM [1-^{14}C] oleoyl-CoA, 1 mM malonyl-CoA, 0.5 mM NADPH, 0.5 mM NADH, 1 mM ATP, 2 mM $MgCl_2$, 1 mM CoASH and 0.08 M HEPES pH 7.2 buffer in a total volume of 0.2 ml.

only C20:1 was formed. On occasion, preparations were obtained in which small amounts of C22:1 were formed.

These results indicated that the two reduction systems (β-ketoacyl-CoA reductase and enoyl-CoA reductase) responsible for converting C18:1 to C20:1 had no clear preference for NADH or NAPDH, whereas one of the reduction systems converting C20:1 to C22:1 show a pronounced requirement for NADPH. These results would suggest, therefore, that at least one of the enzymes for the formation of C20:1 and C22:1 from C18:1 may be dissimilar. The absence of ATP in the system results in a 50% drop in the formation of C20:1 but only a slight drop in C22:1 formation. The role of ATP in the elongation system was not explored further.

Effect of Time on Elongation

Time course experiments showed that in the presence of NADPH and NADH the formation of C20:1 plateaued after 1 hr of incubation; thereafter, a slight decrease in the level of C20:1 with time was noticed (data not shown). On the other hand, elongation of the newly formed C20:1 to C22:1 proceeded relatively slowly and reached a plateau after only 2 hr of incubation. Although the time curve suggests a possible precursor-product relationship between C20:1 and C22:1, the observation that C20:1 did not drop to a lower level as C22:1 was formed could be interpreted as follows: as C20:1-CoA is being synthesized, three different systems would be in competition with this newly formed substrate, namely, the elongation of C22:1-CoA, an acyl-CoA thioesterase and an acyltransferase.

Analyses of radioactivity distribution in different lipid classes in time course experiments showed (i) a decrease in radioactivity in the acyl-CoA fraction and (ii) a simultaneous appearance of radioactivity principally in fatty acid and triacylglycerol fractions and to a very small extent in polar lipids and diglyceride fractions. In both aqueous and organic fractions, percentage distribution of radioactivity among C18:1, C20:1 and C22:1 was similar. These results indicated the presence of broadly specific thioesterase and acyltransferase involved in triacylglycerol biosynthesis and would support the interpretation just presented.

Time course experiments in the presence of NADPH showed that both C20:1 and C22:1 were found (Fig. 1A). However, with incubation involving NADH alone, only C20:1 was formed; little C22:1 was detected even when formation of C20:1 had leveled off (Fig. 1B; see also Table 4).

Effect of Substrate Concentration

At concentrations of malonyl-CoA lower than 25 μM, elongation of [1-^{14}C]C18:1-CoA was not measurable. The elongation activity increased almost linearly with increasing malonyl-CoA concentration. Even at 0.5 mM level, no plateauing of activity was observed (data not shown). The inability of low levels of malonyl-CoA (< 25 μM) to participate in elongation of [1-^{14}C]C18:1-CoA resolved the preliminary observation that a low level of [2-^{14}C] malonyl-CoA (17 μM) was ineffective in elongating unlabelled C18:1-CoA. This observation would suggest that the elongating system may not be readily accessible to malonyl-CoA in that it may be deeply inbedded in the lipid bilayer.

With varying concentrations of oleoyl-CoA at 0.5 mM malonyl-CoA, the elongation activity increased linearly up to 30 μM of oleoyl-CoA; above this concentration, the activity began to level off (data not shown). Michaelis-Menten constants were not determined because of the errors inherent with the use of crude extracts, etc.

Effect of Inhibitors

As expected, trichloroacetate (TCA) significantly inhibited (24) the elongation of oleoyl-CoA; about 1.5 mM of TCA was required for 50% inhibition (Table 2). On the other hand, inhibition by $Na_2S_2O_5$ was much more pronounced; only 0.25 mM caused about 45% inhibition (Table 2). Inhibition of elongation by $Na_2S_2O_5$ has not been reported before. It also was observed that at lower levels, both TCA and $Na_2S_2O_5$ preferentially inhibited C22:1

TABLE 2

Inhibitory Effect of $Na_2S_2O_5$ and TCA on Elongation of [1-^{14}C]Oleoyl-CoA by Cell-Free Extracts of *Brassica juncea* Seeds

	FA formed (nmoles)hr/ml extract	
	C20:1	C22:1
No inhibitor	11.1	4.9
$Na_2S_2O_5$		
0.25 mM	8.7	0.0
0.5 mM	4.7	0.0
1.0 mM	3.6	0.0
2.0 mM	1.3	0.0
TCA		
0.25 mM	10.1	3.3
0.5 mM	12.1	0.0
1.0 mM	9.9	0.0
2.5 mM	4.9	0.0

Experimental procedures are described in the Materials and Methods section.

formation (Table 2). This result indicates the possibility that in the elongation of C18:1-CoA to C22:1 at least one enzyme involved in the conversion of C20:1 to C22:1 is sensitive to inhibition by these compounds at lower concentrations.

Subcellular Localization of C18:1 Elongation Activity

The isolation of the subcellular fractions 2,000 xg, 15,000 xg and 100,000 xg pellets from developing *B. juncea* seeds using conventional techniques was seriously complicated because of the high levels of triglycerides in the seeds. Since subcellular fractions, other than 2,000 xg pellet, invariably contained chlorophyll, additional steps were used to obtain these fractions free from chlorophyll, if necessary, at the cost of quantitative recovery.

Measurement of elongation activity indicated that the 2,000 xg pellet consistently had the highest elongation activity (Table 3); the 100,000 xg pellet was devoid of elongation activity. On the other hand, the 15,000 xg pellet and fat fraction did contain some activity (Table 3). The elongation activity in subcellular fractions was proportional to their chlorophyll content, but independent of their C22:1 content.

These results seem to suggest that the 2,000 xg pellet is the prime site for oleoyl-CoA elongation and the elongation activity found in other particulate fractions may be due largely to their contamination with the 2,000 xg membranes. At the same time, the presence of a substantial amount of C22:1 in the 2,000 xg pellet indicates extensive contamination of this particulate fraction with the fat pad. Therefore, the possibility that elongation of oleoyl-CoA occurs in the fat pad cannot be dismissed. Further work is necessary to develop methods which can obtain membranes clearly separate from the fat pat fraction.

Elongation of Other Acyl-CoAs

In addition to oleoyl-CoA, [1-^{14}C]stearoyl-CoA was elongated to C20:0 and C22:0 by the cell-free homogenate of *B. juncea* in the presence of malonyl-CoA, NADPH and ATP (Table 4). On the other hand, [1-^{14}C]palmitoyl-CoA was poorly elongated. Lipid analyses showed that the acylthioesterase or transferase could not be responsible for very low C16:0 elongation activity (data not shown). These results are different from those obtained for leek epidermis where C16:0- and C18:0-CoAs are elongated, but not C18:1-CoA by epidermal microsomes (17). The results are consistent with the system in *L. alba* seeds (14), where radioactive C18:0 and C18:1 acids, and not C16:0 acid, were elongated.

As expected, [1-^{14}C]-eicosenoyl-CoA was elongated to C22:1 by the cell-free homogenates. The preference of the reductive step in the elongation of C20:1 to C22:1 for NADPH rather than NADH is clearly shown in Figures 1C and 1D.

TABLE 3

Distribution of C18:1 Elongation Activity in Different Subcellular Fractions

Subcellular fractions	Chlorophyll (μg/ml)	Protein (mg/ml)	Relative mass of C22:1	Elongation activity (nmoles of C20:1 + C22:1 formed/hr/ml)
Fat pad	3.4	0.89	1	12.4
2,000 xg pellet	9.6	2.18	1.15	38.8
15,000 xg pellet	0.8	1.06	trace	trace
100,000 xg pellet	0.2	0.51	trace	0.0

Experimental procedures are described in the Materials and Methods section.

TABLE 4

Elongation of Acyl-CoAs Catalyzed by Cell-Free Extracts Prepared from Developing *Brassica juncea* Seeds

	Percentage of Radioactivity in Elongation Products					
Acyl-CoAs	C18:0	C20	C20:1	C22:0	C22:1	C24:0
[1-^{14}C]C18:1-CoA						
NADPH			18.6		15.4	
NADH			19.8		3.6	
[1-^{14}C]C18:0-CoA						
NADPH		30.8		22.9		1.8
NADH						
[1-^{14}C]C20:1-CoA						
NADPH					23.5	
NADH					9.1	
[1-^{14}C]C16:0-CoA						
NADPH	trace	trace				

For C16:0-, C18:0- and C18:1-CoAs, 50 μl of the cell-free extract was incubated at 30 C for 2 hr with 17 μM acyl-CoA, 1 mM malonyl-CoA, 1 mM ATP-Mg^{2+}, 1 mM CoASH and 1 mM NADPH in a total volume of 0.1 ml. For elongation of C20:1-CoA, 0.25 ml of the cell-free extract was incubed with 34 μM of [1-^{14}C]C20:1-CoA in a total volume of 0.5 ml.

CONCLUSION

The results presented in this investigation suggested the following: (i) the site of elongation system(s) appeared to be in the membranes associated with a large organelle (2,000 xg) rather than with a microsomal pellet. (ii) Both oleoyl-CoA and stearoyl-CoA are readily elongated, but palmitoyl-CoA is not. Long chain saturated fatty acids are minor components (< 6% C20:0 and C22:0) in *B. juncea* fatty acid composition. It can thus be suggested that stearoyl-CoA under in vivo conditions is formed at very low levels from the de novo $C_2 \rightarrow C_{18}$-ACP system. Since stearoyl-ACP is so rapidly converted to oleoyl-ACP by the highly specific stearoyl-ACP desaturase, this substrate is never converted to stearoyl-CoA which then could have served as the precursor for the longer chain saturated fatty acids. That palmitoyl-CoA is not elongated is an added guarantee that long chain saturated fatty acids do not accumulate in the seeds of *B. juncea*. (iii) Acyl carrier protein does not appear to be involved in the elongation system. (iv) NADPH can be used as a reductant in both the C18:1 → C20:1 and C20:1 → C22:1 steps, but NADH serves only as a reductant in the C18:1 → C20:1 step; it is far less effective as a reductant for the conversion of C20:1 → C22:1. (v) The elongation of C20:1 → C22:1 is more susceptible to inhibition by sodium metabisulphite and trichloroacetate than the elongation of C18:1 → C20:1.

These results would suggest that the elongase involved in the C20:1 → C22:2 may be more sensitive to these inhibitors as well as displaying a specificity for NADPH. These results are compatible with those obtained from fatty acid analysis of seed oils with a constant level of 11-14% C20:1 but variable amounts of erucic acid in three different phenotypes (C22:1 - 30.4, 20.9 and 12.8%), and in one zero erucic acid phenotype of 0.0% C22:1 and 1% C20:1 (7).

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Modification of Alkenyl Chain Profile in Plasmalogens of Rat Heart Mitochondria by Dietary Trielaidin

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ABSTRACT

Effects of dietary trielaidin upon the alkenyl chain profile of plasmalogens were studied using heart mitochondria of rats fed a semi-purified diet containing 10% of fat supplement in which elaidic acid accounted for 69% of total fatty acids. Alkyl substituted dioxane (ASD) derivatives of the alkenyl groups of plasmalogens were prepared and analyzed by silver nitrate TLC and by GLC on different phases (BDS and OV-275). After two months of feeding the experimental diet, 40% of the ASD contained a *trans*-octadecenyl chain, suggesting that dietary elaidic acid was reduced in vivo to the corresponding alcohol and incorporated into plasmalogens. There was a simultaneous decrease in the percentage of ASD containing saturated chains, but the percentage of ASD substituted with *cis*-octadecenyl chains was not significantly affected. These observations suggested that elaidic acid may compete with saturated fatty acids, but not with *cis*-octadecenoic acids during the plasmalogen biosynthesis. Feeding trielaidin did not seem to have any significant influence on the relative proportions of plasmalogens, which accounted for 11-12%, on a phosphorus basis, of total heart mitochondria phospholipids.

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INTRODUCTION

Due to their presence in partially hydrogenated vegetable oils, *trans* fatty acids are common (1) and significant constituents of the human diet (2). Their nutritional properties and metabolic fate have been the subject of many recent reviews (3-6) and a monograph (7). A great number of studies has been designed to elucidate the effect of *trans* fatty acids upon the fatty acid profile of membrane phospholipids in mammalian cells. Although plasmalogens (1-*O*-(1′-alkenyl)-2-acyl-glycerophospholipids) are major constituents in many biological membranes (8), it appears that these particular components have not been studied extensively in relation to *trans* fatty acid ingestion.

However, it is know that supplementation of *trans*-9-octadecenoic acid (elaidic acid) to cultured LM cells (9) results in an accumulation of elaidyl alcohol. Cell-free systems from the mouse preputial gland tumor are able to promote alcohol synthesis from elaidic acid when adequate cofactors are present (10). On the other hand, radioactive labelled mixtures of isomeric *trans*-octadecenols have been shown (11) to be incorporated into the alkenyl moieties of plasmalogens from L 1210 and S 180 ascites cells or from rat brain after injection. Moreover, isomeric *cis*- and *trans*-octadecenoic acids of dietary origin have been found to be utilized in the biosynthesis of heart plasmalogens (12).

It is well known that dietary elaidic acid is readily incorporated into phospholipids of rat heart or of rat heart mitochondria (13,14). Moreover, it is known that this organ, or its subcellular organelles, are rich in plasmalogens (15). Thus, this investigation was undertaken to detect whether elaidic acid might affect the alkenyl chain composition of the 1-position of this class of phospholipid.

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MATERIALS AND METHODS

Animals and Diets

Male Wistar rats were taken at weaning and divided into three groups. One group of 15 animals was raised for two months on a standard laboratory chow (A-04, UAR, Villemoisson-Sur-Orge) containing 4% fat. A second group of 15 animals was fed for the same period a basic fat-free diet (UAR, Villemoisson-Sur-Orge) supplemented with 10% of a mixture of SO_2 elaidinized triolein and sunflower oil (40:1, by weight). The composition of these diets has been described elsewhere (14). The fatty acid composition of dietary fats is given in Table 1. A third group of 20 rats was used immediately after weaning.

Preparation of Mitochondria

Rats were decapitated and their hearts were quickly excised, washed with cold 0.9% NaCl and placed in ice-cold buffer (10 mM Tris, 0.25 M sucrose, 2 mM EDTA, pH 7.2). Hearts were minced with scissors, pooled, and homogenized in a Potter homogenizer in the presence of type VIII protease from *Bacillus subtilis*

TABLE 1

Fatty Acid Composition of Dietary Fats[a]

Diet[c]	Fatty acids[b]						
	16:0	16:1	18:0	18:1	18:1t	18:2	18:3
Trans-free	19.6	2.7	1.9	19.1	tr.	48.8	3.8
Trielaidin	0.3	N.D.	0.3	27.8	69.0	2.4	tr.

[a]Composition as weight percent of total fatty acids.

[b]Shorthand designation: number before colon = chain length; number after colon = number of double bonds; t = *trans* double bond; tr. = trace amounts, less than 0.2%; N.D. = non-detected.

[c]*Trans*-free = standard laboratory chow, containing 4% (by wt) fat. Trielaidin = mixture of elaidinized triolein and sunflower oil (40:1, by wt) supplemented at a level of 10% (by wt) to a basic fat-free diet.

(Sigma, 0.8 mg protease/g of heart tissue). The homogenate was centrifuged at 600 × g for 10 min to remove cell debris and the supernatant at 6000 × g for another 10 min. The mitochondrial pellet was washed twice and finally resuspended in the homogenizing solution, but without EDTA. Purity of mitochondria was ascertained by known marker enzymes (16).

Extraction of Lipids

The total lipids of mitochondria were extracted according to the procedure of Folch et al. (17) with chloroform/methanol (2:1, v/v) containing 0.02% of 2,6-di-tert-butyl-4-methylphenol (BHT) as an antioxidant. Extracts were dried in a rotary evaporator at 30 C, dissolved in pure chloroform and filtered. After evaporation of the solvent in a stream of nitrogen, the extracts were dissolved in chloroform/methanol (2:1, v/v) and stored at −20 C.

Analytical Procedures

Individual phospholipids were separated by two-dimensional thin-layer chromatography (TLC) on 0.3 mm silica gel H plates using chloroform/acetone/methanol/acetic acid/water (75:30:15:15:7.5, v/v/v/v/v) in the first direction and butanol/acetic acid/water (90:30:30, v/v/v) in the second direction (14). When plasmalogens had to be quantified, a modification (14) of the method of Horrocks (18) was used. After exposure to iodine vapors, spots were scraped. Ashing and phosphorus determination were made according to Ames (19).

Total phospholipids were separated from neutral lipids by preparative TLC in a solvent system containing diethylether/acetone (90:30, v/v) and 0.02% of BHT and extracted from the silica gel by elution with chloroform/methanol (2:1, v/v), methanol and methanol/water (9:1, v/v). A five centimeter migration was sufficient to achieve complete separation.

Alkenyl moieties from total plasmalogens were converted to alkyl-substituted dioxanes (ASD) by reacting total phospholipids with 1,3-propanediol in the presence of 4-toluenesulfonic acid in dry benzene (20). ASD were purified by TLC on silica gel H coated plates using hexane/diethyl-ether/acetic acid (90:10:1, v/v/v) and eluted prior to gas liquid chromatography (GLC). ASD also were separated according to the number and geometry of double bonds by TLC on layers of silica gel H containing 5% silver nitrate by weight. The solvent used was hexane/diethylether/acetic acid (94:4:2, v/v/v). After development, the plates were sprayed with 0.2% 2′,7′-dichlorofluorescein in ethanol and viewed under UV light. Each band was scraped, 1 ml of methanol/water/acetic acid (10:10:1, v/v/v) was added to the gel and ASD were extracted with several portions of hexane. With this procedure, 2′,7′-dichlorofluorescein remained in the lower aqueous phase. Each isolated fraction was analyzed further by GLC.

ASD compositions were determined using Varian 1400 and 940 chromatographs equipped with flame ionization detectors. Analyses were carried out with 300 × 0.32 cm or 600 × 0.32 cm stainless steel columns packed, respectively, with 7% BDS on 100:120 mesh gaschrom Q, at 185 C, or 15% OV-275 on 80:100 mesh chromosorb P-DMCS, at 215 C.

The identification of ASD was made by comparing their relative retention times with those of the corresponding methyl esters (21) on a BDS column. Peak areas were calculated by chromatograph-linked integrators (Varian CDS 110 and Shimadzu ICR-1-B).

RESULTS

Plasmalogen Composition

Choline glycerophospholipids and ethanolamine glycerophospholipids were the two main components of heart mitochondria from rats fed a standard chow diet. Their proportions were about the same, that is 39 ± 1% of total phospholipids. 1-*O*-(1′-Alkenyl)-2-acyl glycerophosphocholine (choline plasmalogen) and 1-*O*-(1′-alkenyl)-2-acyl glycerophosphoethanolamine (ethanolamine plasmalogen), together, represented 11-12% of total phospholipids. Ethanolamine plasmalogens accounted for 25% of ethanolamine glycerophospholipids and choline plasmalogens for 3 to 4% of choline glycerophospholipids.

The addition of trielaidin to the diet had no effect on the relative proportion of ethanolamine plasmalogens which represented 24.5 ± 1% of ethanolamine glycerophospholipids. A similar percentage was observed in heart mitochondria of rats sacrificed at weaning.

In all cases, choline plasmalogens remained at low levels, not exceeding 5% of choline glycerophospholipids.

Alkenyl Chain Profile

Unfractionated ASD prepared from total plasmalogens from heart mitochondria of rats taken at weaning or fed a standard chow diet showed three main peaks upon GLC analysis on a BDS column. These were identified (21) as corresponding to 16:0, 18:0 and 18:1 chains. The ratio of their retention times to the retention times of the corresponding fatty acid methyl esters was found to be 2.60 ± 0.03 in our experimental conditions. On this basis, minor peaks were attributed to 17:0 and 18:2 chains. The same three main peaks were present when ASD prepared from trielaidin fed rats were analyzed on the same type of column, but with a different profile. Analysis by GLC on an OV-275 column of ASD prepared from animals fed *trans*-free diets also showed three major peaks. The extra peak was eluted between peaks corresponding to 18:0 and *cis*-18:1 chains.

ASD prepared from heart mitochondria plasmalogens of rats fed the standard diet were resolved in two bands after silver nitrate TLC fractionation. ASD from heart mitochondria plasmalogens of rats fed the trielaidin supplemented diet showed the same two bands and an additional one migrating between them. Analysis by GLC on a BDS column of ASD eluted from common bands revealed that the fast migrating components (r_f = 0.34) corresponded to saturated chains of mainly 16 and 18 carbon atoms. Analysis of the slow migrating components (r_f = 0.23) indicated that these ASD contained almost exclusively *cis*-monounsaturated chains derived from 18 carbon atom alkenyl chains. As a shoulder was seen on the descending portion of the peak, it could be deduced that more than one positional isomer was present.

When ASD from the intermediate band (r_f = 0.28) were analyzed on a BDS column, we could observe one peak with the same retention time as the major *cis*-18:1 chain peak from the slow migrating band. But upon GLC analysis on an OV-275 column, this component gave a peak having the same retention time as the supplementary peak observed on chromatograms of unfractionated ASD from trielaidin fed rats. This peak was clearly distinct from the peak given by isolated *cis*-18:1 chains.

It is known that *trans*-monounsaturated fatty acid methyl esters, upon silver nitrate TLC fractionation, have an intermediate r_f between saturated and *cis*-monounsaturated chains (22). Upon GLC analysis on columns packed with BDS, a *trans*-monounsaturated chain is not separated from its *cis*-isomer (23), but on columns packed with OV-275, the *trans*-monounsaturated component is eluted before the *cis* isomer (24).

Thus, according to its chromatographic behavior, and by analogy with what is known for fatty acid methyl esters, it could be deduced that the unknown component present in ASD prepared from plasmalogens of rats fed a trielaidin rich diet had an 18 carbon chain and contained one *trans* double-bond.

The position of the *trans* double-bond was not established experimentally. But as the sole source of *trans* double-bond was trielaidin in the diet, it seemed reasonable to assume that the *trans* double-bond was located between the 9 and 10 positions.

In Table 2 are compared the alkenyl chain compositions, as weight % of their ASD derivatives, of plasmalogens from heart mitochondria of rats at weaning or fed for two months either a standard chow diet or the experimental trielaidin containing diet.

For rats taken at weaning or raised on a standard chow, the main alkenyl moiety of plasmalogens was a 16:0 chain, which represented about one half of total alkenyl chains. The two other major components were 18:0 and *cis*-18:1 chains. Minor components included 18:2 and 17:0 chains. In the case of animals fed a trielaidin enriched diet, the level of alkenyl chains containing a *trans* double-bond was very high (40.9%). Plasmalogens from this group, as compared to the *trans*-free

TABLE 2

Alkenyl Chain Composition of Total Plasmalogens from Rat Heart Mitochondria[a]

	ASD[b]					
Diet	16:0	17:0	18:0	18:1c	18:1t	18:2
At weaning	53.5	1.2	18.1	23.2	N.D.	3.7
Trielaidin	24.5	tr.	7.8	25.5	40.9	tr.
Trans-free	46.2	1.4	20.3	27.7	N.D.	4.3

[a]Composition as area percent of ASD derivatives. Values are means of duplicate analyses of two preparations of ASD in each case. Standard deviations were less than 5%.

[b]Same shorthand designation as for fatty acids, but the number before colon refers to the number of carbons of the alkyl substituent of ASD plus one. For other meanings, see notes to Table 1.

groups, contained only 32.3% of saturated alkenyl chains, instead of 67.9% (rats raised on a standard diet) or 72.8% (rats taken at weaning). All of the saturated species, including 17:0 chains, were decreased. The level of *cis*-octadecenyl chains did not seem to be affected by diets and remained fairly unchanged, around 25% for the three groups. These observations suggested that more than three-quarters of the incorporated *trans*-octadecenyl chains displaced saturated species. The 18:2 chains represented approximately 4% of the alkenyl chains of plasmalogens from weanling rats or from animals fed a standard laboratory chow, but only trace amounts were detected in trielaidin fed rats. Disappearance of 18:2 alkenyl chains in plasmalogens from heart mitochondria also was observed when the animals were fed the basic fat-free diet for one month (unpublished data).

DISCUSSION

Phospholipids from heart mitochondria of rats are composed mainly of phosphatidylcholine, phosphatidylethanolamine and cardiolipin in the approximate ratio 2:2:1 (on a phosphorus basis) (14,20). Similar compositions have been reported for heart mitochondria of other mammals (25,26). Plasmalogens of rat heart mitochondria are 90-95% of the ethanolamine form (14,20). Choline plasmalogens are only minor components in contrast to other mammals (8,16). According to some recent results, *trans* fatty acids from hydrogenated oils (13, 27) do not modify the relative percentages of these phospholipid classes in rat heart mitochondria. Our results agree with the observations in that the plasmalogen content of rat heart mitochondria seems to be rather insensitive to the very high level of trielaidin used in the experimental diet.

The alkenyl composition of plasmalogens in most normal rat tissues consists mainly of 16:0, 18:0 and *cis*-18:1 chains (28). In heart plasmalogens, the 18:1 chains are composed of the *cis*-9 and *cis*-7 isomers (12). This is probably the reason why the *cis*-monounsaturated ASD were eluted as an asymmetrical peak during GLC analysis.

There is some disagreement concerning the presence of 18:2 alkenyl chains in rat tissue plasmalogens (28,29), which may be confused with 19:0 chains during GLC analysis of their derivatives (20,28). The saturated fraction isolated by silver nitrate TLC did not show any peak corresponding to an ASD derived from a 19:0 alkenyl chain. A faint band could be observed occasionally beneath the band containing *cis*-monounsaturated ASD after silver nitrate TLC fractionation. This band was shown to contain the component tentatively identified as 18:2 ASD by GLC analysis. Thus, it seems that plasmalogens from heart mitochondria contained 18:2 alkenyl chains rather than 19:0 chains. This conclusion also is supported by the fact that this component was almost absent in plasmalogens of rats which were raised on a basic fat free diet, devoid of linoleic acid.

The general pattern of alkenyl chain distribution in plasmalogens seems to be due primarily to the specificity of the microsomal oxido-reductase which catalyzes the reduction of fatty acids, as their coenzyme A derivatives, to the corresponding alcohols (30). This fatty acid reducing system has been shown, in vitro, to have a great specificity for palmitic, stearic and oleic acids (31,32), although some activity toward linoleic acid also has been observed (32). As *cis*-11-octadecenyl chains also are found in plasmalogens from different animal origin (12,33), it may be deduced that *cis*-vaccenic acid also is a good substrate for the microsomal reductase. The in vitro conversion of elaidic acid to the corresponding alcohol also has been reported (9,10). All positional

isomers of *cis*- and *trans*-octadecenoic acids may be substrates for the in vivo plasmalogen biosynthesis (12). However, it seems that there are no data available on the relative specificity of these enzymatic systems toward mixtures of saturated and *cis*- and *trans*-octadecenoic acids. Our observations suggest that dietary elaidic acid may compete with palmitic and stearic acids, resulting in an almost quantitative replacement of saturated alkenyl chains by *trans*-9-octadecenyl chains in plasmalogens.

Replacement of saturated fatty acids by *trans* fatty acids in phospholipids is known to occur in membranes of cultured cells (34). Similar observations have been made with intact animals fed diets supplemented with *trans* fatty acids (35). We have shown recently that this was the case for individual phospholipids from inner and outer membranes of rat heart mitochondria (14). Our present observations indicate that a similar replacement of saturated chains by *trans*-monounsaturated chains occurs at the 1-position of plasmalogens. This probably is due to the fact that *trans*-monounsaturated and saturated hydrocarbon chains have similar linear geometrical structures, easily confounded by enzymes but distinguished from the bent shape of *cis*-monounsaturated chains. Finally, the disappearance of 18:2 alkenyl chains from plasmalogens has rather to be linked to the low level of linoleic acid in the diet (2.4% of total fatty acids) than to the large amount of elaidic acid. As indicated, the same phenomenon could be obtained by feeding rats a fat free diet.

As the physiological significance of plasmalogens is still obscure, it would be purely speculative to draw a conclusion about the biological impact of the modifications induced by dietary trielaidin. Moreover, it must be emphasized that the design of our experiment was made to amplify any effect of elaidic acid. The high level of the unusual triglyceride, trielaidin, added to the diet was not realistic and it seems improbable that these conditions would occur in human diets. Nevertheless, some reports have given partial evidence that in membranes, *trans* fatty acids have a physical behavior somewhat similar to saturated fatty acids (34,36). If these observations can be extended to alkenyl chains, the replacement of saturated chains by *trans*-monounsaturated chains may not lead to gross modifications of the membrane "fluidity" and changes in its biological activities. Finally, our observations indicate that previously measured incorporation of elaidic acid in plasmalogen-containing organs probably was underestimated unless alkenyl chains of plasmalogens were taken into account.

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New Long-chain Hydroxy Acids from *Grevillea decora*

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ABSTRACT

In addition to the ω-5 olefinic acids found in other *Grevillea* species, about 10% of the acyl groups of *G. decora* seed oil contain a hydroxy group and an ω-5 double bond. The chainlengths of these acids are from C_{22} to C_{30}, with the largest concentration at the C_{26} and C_{28} chainlengths. The hydroxy group is located on odd carbons from carbon-5 through carbon-13. These acids previously were unknown in nature. The most abundant of these are 7-hydroxy-*cis*-17-docosenoic, 7-hydroxy-*cis*-19-tetracosenoic, 9-hydroxy-*cis*-19-tetracosenoic, 9-hydroxy-*cis*-21-hexacosenoic, 11-hydroxy-*cis*-21-hexacosenoic, and 13-hydroxy-*cis*-23-octacosenoic acids. The oil also contains the largest known concentration of the unoxygenated C_{26} and C_{28} ω-5 monoenes.
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INTRODUCTION

It has long been known that certain plants, generally in the Proteaceae family, produce seed oils that contain acyl groups with ω-5 unsaturation (1-3). These fatty acids were reported with chainlengths up to C_{28} (3). In our ongoing study of seed oils from uncultivated plants, we have examined the composition of the seed oil of another Proteaceaous species, *Grevillea decora* Domin, collected in Australia, which also produces ω-5 unsaturated acids. More of the C_{26} and C_{28} isomers are present in this species than have been found in any previously reported Proteaceae. It also biosynthesizes, most likely from the ω-5 unsaturated acids, a family of heretofore unknown hydroxy fatty acids, all containing the ω-5 unsaturation and chainlengths from C_{22} to C_{30}. The position of the hydroxy group varies from carbon-5 to carbon-13, depending on the chainlength involved. Small amounts of C_{22} to C_{26} saturated hydroxy acids also were detected by GC-MS. Long chain (C_{22}-C_{26}) hydroxy acids have been found in a variety of plant materials and fungi (1,4,5). Those of plant origin have been reported to have chainlengths as long as 24 carbons and as many as three hydroxyl groups, but none have been found as long as those present in *G. decora*.

EXPERIMENTAL

Seed was cleaned and ground as previously described (6). Oil was obtained by hexane extraction of the ground seed in a Butt extractor. Infrared analysis was done with a Perkin-Elmer 137 spectrometer either on sodium chloride discs or in chloroform solution. Proton magnetic resonance (PMR) spectra were obtained from $CDCl_3$ solutions in a Bruker WM 300 WB spectrometer. Optical rotation was determined in a Perkin-Elmer model 241 polarimeter. Observations were recorded at 589, 578, 546, 436 and 365 nM at a concentration of 525 mg of ester/100 ml of methanol. Acyl groups were converted to methyl esters by using 10% BF_3 in methanol (7). Esters were isolated either by preparative thin-layer chromatography (TLC) on silica gel plates [(hexane/diethyl ether, 60:40) as developing solvent] or by gravity flow silica columns (hexane/ether, 90:10) to elute the unoxygenated esters and then hexane/ether (60:40) to recover the hydroxy esters. The hydroxy ester fraction was separated further by chain length by means of high performance liquid chromatography (HPLC). A reverse phase Partisil M9 ODS-2 column (Whatman), with acetonitrile/acetone (2:1) (at a flow rate of 5 ml/min) as the mobile phase, was used for this separation. Esters were analyzed by gas chromatography (GC) on both packed and capillary columns. In packed columns the esters were analyzed in a Hewlett-Packard model 7610 gas chromatograph. A 0.9 m × 2 mm glass column packed with 5% Apiezon L and a 3.3 m × 2 mm glass column packed with 5% LAC-2-R 446 were operated isothermally at 190 C. A Packard model 428 gas chromatograph was used with a 50 m by 0.2 mm OV-1 fused silica column for capillary analysis. The column oven was temperature programmed from 160 to 270 C at 2 C/min. The column flow was 1.5 ml/min, and the split ratio 170:1. Equivalent chain lengths (ECL) were determined by using saturated even chain fatty esters from C_6 to C_{28} as standards.

Mass spectrometric analysis was accomplished on either a Kratos MS-30 or a Finnigan/MAT 4535/TSQ. The MS-30 was used with packed 1.3 m × 2 mm OV-1 columns, temperature programmed from 100 to 260 C, and was scanned from 700 to 27 m/z at 3 sec/decade. With the Finnigan/MAT 4535/TSQ the samples

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were introduced into the mass spectrometer by a directly coupled 15 m by 0.25 mm DB-1 fused silica capillary column (J & W Scientific, Rancho Cordova, California). The GC was temperature programmed from 180 C to 300 C at 4 C/min. The injection port temperature was 270 C. The sample was injected into the GC in the split mode, with approximately a 50:1 split ratio. In full scan experiments, the mass spectrometer was scanned from 40 to 700 in 1 second. In multiple ion detection experiments, six selected masses were monitored for 0.025 sec each, for a total scan time of 0.182 sec.

Double bonds were located by methoxylation of the unsaturated hydroxy esters with mercuric acetate in methanol followed by GC/MS (3). Hydroxyl groups were converted to trimethylsilyl derivates with Hydrox-Sil (Regis Chemical Co.).

RESULTS AND DISCUSSION

G. decora lipids were examined in detail because during routine screening the oil displayed a weak absorption band at 3500 cm^{-1} in the infrared. spectrum. TLC of the oil revealed two major components, one migrating as normal triglycerides and the other as monohydroxy triglycerides. Polar components also were observed in the TLC examination of the methyl esters derived from the oil. These observations suggested the presence of some hydroxyl-containing components. On the polar LAC-2-R 446 and the non-polar Apiezon L columns, GC indicated the same ω-5 monoenes that were found in *Grevillea robusta* (3). These components had ECL's 0.3 units less than their saturated analogues on the Apiezon L column and 0.5 units greater on the LAC-2-R 446 column. Chainlengths of the ω-5 monoenes ranged from C_{14} to C_{26}. In addition, long chain hydroxyl monoenoic and saturated esters were observed. In order to resolve the complex nature of the acyl mixture, several strategies were used, i.e. use of capillary GC, separation of esters by polarity and GC/MS.

Capillary GC analysis with the 50 m OV-1 column revealed four series of esters: 1) saturates; 2) Δ-9 unsaturates; 3) ω-5 unsaturates, and 4) ω-5 monoenoic hydroxy esters. The usual monoene had ECL's 0.3 units less than the saturate standards, while the C_{18} diene was 0.4 units less. The ω-5 unsaturated esters were 0.1 to 0.15 ECL units less than the saturates, and the ω-5 unsaturated hydroxy esters had ECL's 1.7 units greater than their saturated analogues. Analysis of this complex mixture was simplified somewhat by separating the more polar hydroxy esters from the unoxygenated esters by TLC and analyzing each fraction separately. GC/MS analyses of these fractions revealed some minor components that were saturated hydroxy esters. The total composition is summarized in Table 1.

In order to positively characterize the hydroxy fatty acids, 200 mg of the more polar esters were recovered from a gravity fed liquid chromatographic silica column. A portion of this fraction was silylated and examined by GC/MS, for which the MS-30 mass spectrometer and a packed OV-1 column in the gas chromatographic inlet were used. Four major hydroxy components were observed, C_{22}, C_{24}, C_{26} and C_{28}. Table 2 outlines the major ions and their intensities resulting from mass spectrometric analysis of these esters by the MS-30. The most abundant ion in the spectra of all the silylated hydroxy esters was m/z 73. Several of these esters exhibited weak molecular ions. The 73 and molecular ions are not included in Table 2. It is apparent from the mass spectra (Table 2, Fig. 1a) that the most common position of the hydroxyl group (as its silyl ether) is in the ω-16 position (m/z 311). Other ions, such as 339 and 367 (Table 2), indicate the presence of additional hydroxy positions at ω-18 and ω-20, the

TABLE 1

Fatty Acid Composition of *Grevillea decora* Seed Oil

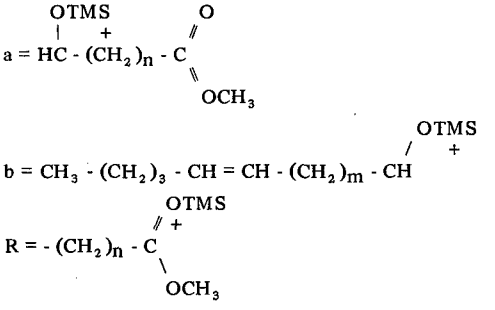
Fatty acid	%, by GC	Fatty acid	%, by GC	Fatty acid	%, by GC	Fatty acid	%, by GC
14:0	0.4	18:0	1.7	22:1	0.1	22:1ω5-OH	0.6
14:1ω5	3.0	18:1	28.9	22:1ω5	1.0	24:0-OH	tr
15:0	0.1	18:1ω5	2.0	24:0	0.9	24:1ω5-OH	0.4
15:1ω5	tr	18:2	1.1	24:1	0.6	26:0-OH	tr
16:0	4.7	18:3	0.2	24:1ω5	2.0	26:1ω5-OH	2.2
16.1	0.5	20:0	0.2	26:0	0.5	28:1ω5-OH	6.2
16:1ω5	21.4	20:1	0.4	26:1ω5	11.2	30:1ω5-OH	tr
17:0	0.5	20:1ω5	1.6	28:1ω5	6.9		
17:1	0.4	22:0	0.2	22:0-OH	tr		

TABLE 2

Major Diagnostic Mass Spectral Fragments of Silylated Esters

Chainlength	(m/z [% of base peak]) M-15	M-90	a	b	R	Hydroxyl location
22	425 (5)	350 (9)	231 (90)	311 (56)	202 (13)	7
24	453 (5)	378 (9)	259 (64)	311 (46)	230 (7)	9
			231 (38)	339 (19)	202 (5)	7
26	481 (4)	406 (10)	287 (79)	311 (64)	258 (14)	11
			259 (12)	339 (5)	230 (2)	9
			231 (7)	367 (2)	202 (1)	7
28	509 (3)	434 (10)	315 (78)	311 (74)	286 (16)	13
			287 (8)	339 (3)	258 (1)	11
30	N/D	N/D	343 (75)	311 (60)	314 (11)	15

N/D = not detected.



latter in the case of the C_{26} esters. These locations indicated by the "b" fragments (Table 2) are confirmed by the "a" fragments and the rearrangement ions (8,9) or "R" fragments.

Since these are new esters and have not been analyzed by mass spectrometry before, the possibility exists that these ions do not represent different hydroxyl positions, but are really fragment ions from the same ester. Capillary GC alone afforded no observable resolution of components within a single chainlength and, therefore, could not disprove this possibility. However, by using the rapid scanning capability of the quadrupole mass spectrometer in combination with the resolution of capillary GC, we showed that these different hydroxy esters do, indeed, exist because they elute from the GC at different retention times. By scanning through specific m/z regions, and not through the entire mass range, increased sensitivity could be achieved. When the C_{24} silyl ethers eluted, m/z 231 and 259 were observed. The 231 ion, representing the hydroxyl in the carbon-7 position, maximized at scan number 5298. With the 259 ion, representing the carbon-9 hydroxy isomer, the maximum occurred during scan 5279. These two components were only slightly resolved when the total ion curve was observed. In this same way isomers with hydroxyls on carbons-5, 7, 9 and 11 were detected in the C_{26} hydroxy esters and the 7, 9, 11 and 13 isomers in the C_{28} esters. The results of this experiment are summarized in Table 3. The terminal end of the molecule also was observed, thus confirming the structural assignments. When the ions that would result from the saturated analogues were monitored, i.e. 313 instead of 311 and 341 instead of 339, we indeed found another set of peaks well resolved from the more abundant unsaturated hydroxy esters. Of course, the ions representing the saturates were found in the unsaturates as well, from naturally occurring isotopes of the more abundant unsaturates. But when these ions were plotted, distinct peaks following the unsaturated peaks at the expected retention times for saturates on a nonpolar GC column were observed. The traces of saturated hydroxy esters found in this manner are reported in Table 1.

The double bond positions in the hydroxy esters were established by the same methoxylation procedure that was used in locating these functional groups in the monoene esters (3). The diagnostic ions of m/z 101 and 115 (two methoxy isomers are formed from each double bond) define the position as ω-5. Corroborating ions, such as 457, 471 and 367 (457-90) and 381 (471-90) are shown in Fig. 1b, which is the

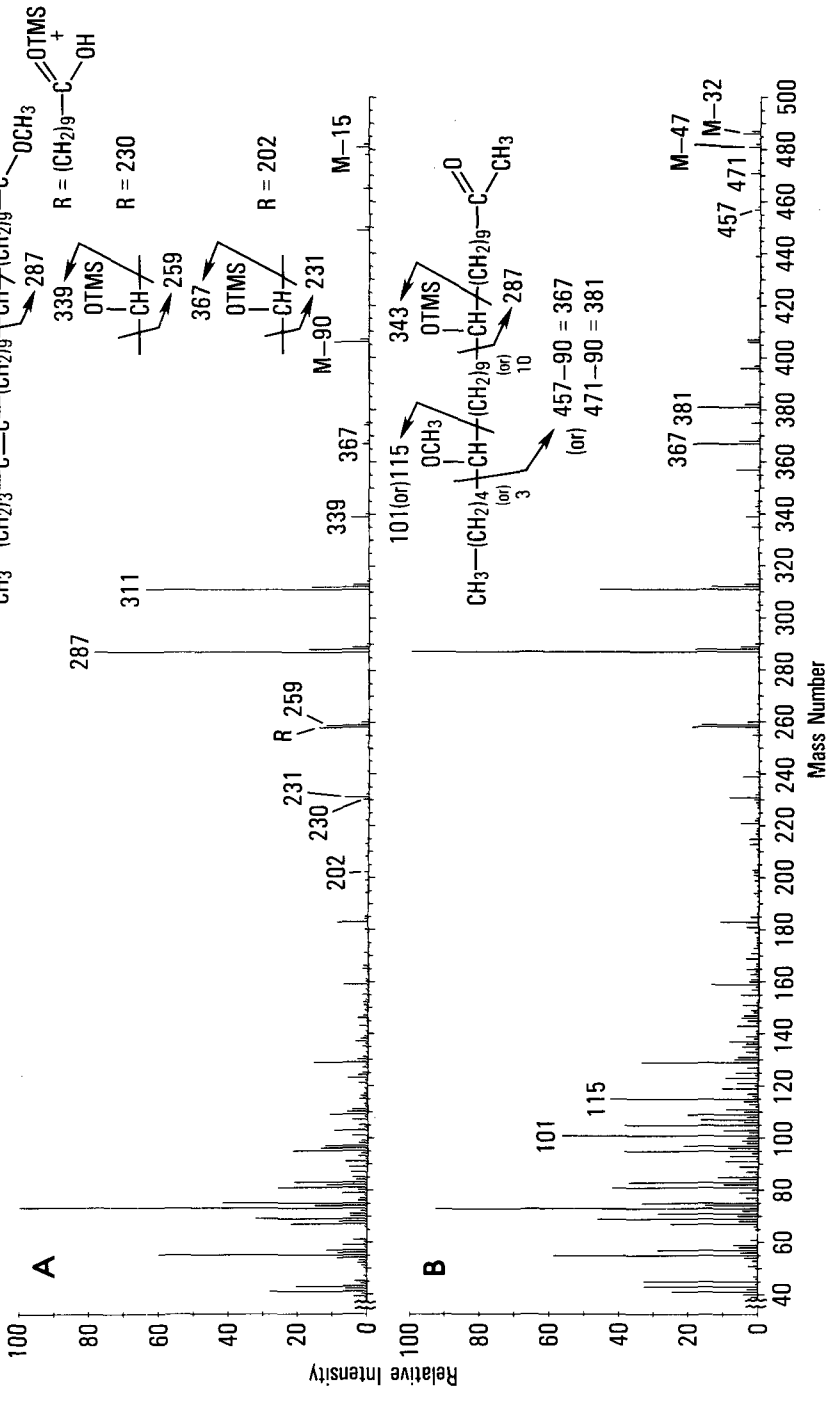


FIG. 1. Electron impact mass spectra of (A) methyl trimethylsilyloxy-*cis*-21-hexacosenoate and (B) the methoxy derivative of (A).

TABLE 3

Location of Hydroxyl Groups

Chainlength	Location (carbon)	m/z	Maximum intensity	Scan number
24	7	231	4416	5298
	9	259	3856	5279
26	5	203	2052	6351
	7	231	7560	6320
	9	259	8640	6295
	11	287	49088	6290
28	7	231	1272	7318
	9	259	1758	7287
	11	287	17536	7276
	13	315	79872	7270

mass spectrum of the methoxylated C_{26} isomer. The very low ion abundance of m/z 343 is due to its loss of methanol resulting in m/z 311. This facile loss of 32 mass units has been noted in similar compounds (10). Mass spectrometry of methoxylated hydroxy esters of other chain lengths resulted in similar spectra.

The individual hydroxy esters were isolated (> 95% pure by GC) by HPLC so that PMR and optical rotational analysis could be performed. Results from the PMR analysis of the C_{28} isomer, used as an example, are consistent with the long chain hydroxy monoenoic ester structure obtained from the mass spectral data. The PMR analysis showed absorbances with chemical shifts (ppm) representing CH_3- (0.9), -CH_2- (1.3), methylene adjacent to the double bond (2.0), methylene adjacent to the carboxyl group (2.3), methine (3.6), methyl ester (3.7) and olefinic group (5.3). The coupling constant for the protons of the double bond is 10.8 Hz, confirming the *cis* geometry (11) inferred from the absence of *trans* in the infrared. No optical rotation was observed at any wavelength. Whether this is because the hydroxy esters are racemic or the specific rotation is too small to measure is not known. However, the latter is quite possible because the hydroxyl group is generally in the center of a long chain molecule making the chiral constituents very similar. The small rotation of saturated hydroxy acids has been previously reported (12).

In conclusion, we have found a series of new fatty acids with chainlengths of 22 to 30 carbon atoms, one hydroxyl group at an odd carbon from carbon-5 through carbon-13, and a *cis* double bond in the ω-5 position. Additionally, trace amounts of saturated analogues were found. This complex mixture most likely resulted from hydroxylation of the unoxygenated analogues that are present in the oil in somewhat greater amounts.

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METHODS

Subfractionation and Characterization of Native and Incubation Enlarged Human Plasma High Density Lipoprotein Particles by High Performance Gel Filtration

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ABSTRACT

Incubation of human plasma in vitro at 37 C results in an increase of the mean particle size of the high density lipoproteins (HDL) accompanied by an almost complete disappearance of the original particles present prior to incubation.

A rapid high performance gel filtration technique has been developed in order to study the chemical composition of subfractions of native and incubation enlarged HDL particles as a function of particle size. Subfractionation of HDL isolated by preparative ultracentrifugation from 3 normal human plasmas incubated in vitro at 0 and 37 C for 24 hr have been performed using a 150 cm long TSK-G 3000 SW column. The separation time was less than 65 min.

The curves obtained at high performance gel filtration of HDL, by monitoring the effluents from the column at 280 nm, agreed well both in positions of peak maxima and relative peak intensities with the particle distribution patterns observed at polyacrylamide gradient gel electrophoresis of the corresponding HDL preparations run in parallel. The different HDL particle subfractions of the effluents from the gel filtration column have been characterized by quantification of free and esterified cholesterol, total phospholipids and apolipoprotein A-I and A-II.

The incubation enlarged HDL particles, subfractionated by the high performance gel filtration technique, were found to have a composition which differed from that of native HDL particles of corresponding size. Incubation enlarged HDL had a generally higher and almost constant relative cholesteryl ester content over the whole particle range compared to native HDL in which a continuous increase in relative cholesteryl ester content could be observed when going from large to small particles.

The molar ratio of phospholipids to free cholesterol was higher in small native HDL particles than in the corresponding large ones. The relation between apolipoprotein A-I and A-II remained nearly constant between small and large HDL particles in each subfractionation experiment. The results demonstrate that the high performance gel filtration technique is a rapid and reproducible means for studying the composition of subfractions of HDL particle populations.

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INTRODUCTION

The high density lipoproteins (HDL) are assumed to circulate in human plasma as globular particles of different sizes with masses between 175 and 350 kilodalton (1). In early ultracentrifugal experiments two density populations of HDL were obtained, denoted HDL_2 and HDL_3 with density regions between 1.063-1.125 and 1.125-1.210 kg/l respectively (2). The idea that a high plasma HDL cholesterol concentration might function as a protective factor for the development of arterial disease has initiated extensive studies of HDL_2 and HDL_3 concentrations in plasma in different clinical conditions. Recently, qualitative analyses of the heterogeneity of HDL as a function of particle size have been performed by means of the polyacrylamide gradient gel electrophoresis technique (3). Using this method, it has been demonstrated that the high density lipoproteins in plasma, collected and immediately stored in ice water at 0 C, increased in particle size during incubation in vitro at 37 C (4-6). Earlier studies, using ultracentrifugation, also had indicated that incubation at 37 C modified HDL (7,8).

In order to study the metabolic role of this HDL particle size heterogeneity and interconversion of small to large particles in normal and hyperlipidemic plasma, it seems necessary to quantify the components of the different HDL particle populations. The polyacrylamide gradient gel electrophoresis technique, however, does not allow standard chemical and immunological methods to be used for the quantification of lipids and apolipoproteins in the zones corresponding to different HDL particle subfractions.

The present report describes a rapid and reproducible high performance gel filtration

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technique for particle size subfractionation of native and enlarged HDL isolated by preparative ultracentrifugation from human plasma incubated at 0 and 37 C respectively. The different HDL subfractions have been characterized by quantification of their content of free and esterified cholesterol, total phospholipids and apolipoprotein A-I and A-II. The results are reported herein.

EXPERIMENTAL PROCEDURES

General Methods

Protein was determined according to Lowry (10), using bovine serum albumin (Fraction V, powder, Sigma Chemical Company, St. Louis, Missouri) as protein standard. The lipids of HDL were extracted with chloroform-methanol (11), and phospholipids (12) were estimated on aliquots of the chloroform phase. Free and esterified cholesterol were determined enzymatically (13) directly on aliquots of HDL using Merck kits 14106, 14107 and 14108 (E. Merck, Darmstadt, W. Germany). Cholesterol analyses were made immediately after column separation of HDL in order to minimize the risk of autoxidation of HDL cholesterol in the diluted solutions. Polyacrylamide gradient gel electrophoresis was carried out as previously described (14) using the PAA 4/30 gel (Pharmacia Fine Chemicals, Uppsala, Sweden). Analytical polyacrylamide gel isoelectric focusing in 8M urea of solubilized apolipoproteins of HDL was performed as described earlier (15,16). The apolipoproteins of HDL were solubilized in aqueous isopropanol (17) after dilution of the protein concentration of the HDL preparations to about 0.7 mg/ml. Phospholipids were removed by one extraction with two volumes of ethyl ether.

Collection and Incubation of Plasma

Venous blood was collected from fasting normolipidemic subjects, not acutely ill or on chronic medication, into EDTA-tubes, which were immediately placed in an 0 C ice water mixture. Plasma was then isolated without delay by low-speed centrifugation at 1 C. One aliquot of the plasma sample was stored in ice water for 24 hr, and another portion was incubated for the same time at 37 C in N_2-atmosphere. After completed incubation all plasma samples were put into ice water.

In one series of experiments, VLDL and LDL were first removed from plasma before incubation at 0 and 37 C in the following way. The density of 0 C cold plasma (5 ml) was raised to 1.070 kg/l by dissolving solid sodium bromide (402.5 mg) in a 6.5 ml Beckman Ultra-Clear® centrifuge tube. The tube was filled with 0 C cold sodium bromide solution (d = 1.070 kg/l) and capped. When the salt in the tube had dissolved, the content was mixed to homogeneity. Ultracentrifugation was then carried out at 1 C and 40,000 rpm for 20 hr as described below. The top fraction containing VLDL and LDL was recovered by tube slicing and discarded. The bottom fraction was dialyzed overnight at 1 C against 0.15 mol/l sodium chloride pH 7.0 with occasional changes of salt solution. This VLDL and LDL depleted plasma sample was used for incubation and subsequent isolation of HDL in one series of gel filtration experiments.

Isolation of HDL from Plasma

After the incubation of plasmas, HDL was isolated between the densities 1.063 and 1.210 kg/l in a Beckman Model L5-75 ultracentrifuge using the 50.3 Ti rotor at 1 C and 40,000 rpm by the following procedure.

The density of the plasma, temperature equilibrated at 0 C with ice water, was raised to 1.063 kg/l by the addition of solid sodium bromide (73.4 mg/ml). Beckman Bell-top ultracentrifuge tubes (6 ml Recorder No. 344320) were filled with plasma treated in this way, sealed and centrifuged for 20 hr. The tubes were then cut with a Beckman Tube Slicer to give a bottom fraction of 3.35 ml. The top fraction, containing VLDL and LDL, was discarded.

The density of the bottom fraction was raised to 1.210 kg/l by the addition of solid sodium bromide (180 mg/ml). A Bell-top tube, described above, was filled with this sample and centrifuged for 65 hr. Finally a top fraction of 1 ml containing all HDL was harvested by tube slicing. In some experiments the HDL preparation was diluted with 0 C cold sodium bromide solution (d = 1.210 kg/l). Collected HDL preparations were put immediately into a 0 C cold ice water mixture.

The HDL fractions isolated by this technique contained 6.2 to 18.7 mg protein/ml and were used directly without being dialyzed for polyacrylamide gradient gel electrophoresis and gel filtration experiments.

Determination of Apolipoproteins

Highly purified apolipoprotein A-I and A-II for primary standards and antibody preparations were obtained from HDL isolated by preparative ultracentrifugation (18), by means of selective extraction with isopropanol-ethyl ether (17,19) followed by preparative isoelec-

tric focusing of the lipid-free apolipoprotein mixture on Sephadex G-200 in 8M urea as described previously (20). The A-I and A-II preparations were free of apolipoprotein E, C-I, C-II and C-III as analyzed by two-dimensional immunoelectrophoresis (15), double immunodiffusion in Ouchterlony plates, SDS-polyacrylamide gel electrophoresis according to Laemmli, and by analytical polyacrylamide gel isoelectric focusing in 8M urea as described above.

Monospecific antibodies against A-I and A-II were raised in albino rabbits as described in detail earlier (21). Apolipoprotein A-I and A-II in the isolated HDL samples were determined by electroimmunoassay using the monospecific antibodies described above and essentially as recommended by Alaupovic et al. (22). The concentration of anti A-I and A-II (precipitated immunoglobulins) in the agarose plates (10 × 10 cm) was 50 and 120 $\mu g/cm^2$ respectively.

As secondary antigen standard, an HDL preparation was used which had been isolated by preparative ultracentrifugation. The apolipoprotein content of this standard preparation also was determined after quantitative solubilization of the apolipoproteins in aqueous isopropanol (17,19).

High Performance Gel Filtration of HDL

The subfractionation of HDL isolated by ultracentrifugation was performed on a 7.5 × 1500 mm TSK-G 3000 SW column (Toyo Soda Co., Japan, purchased from LKB Sverige AB, Sweden) built up of one 7.5 × 300 mm and two 7.5 × 600 mm columns connected in series. Connections between the columns were made with 40 mm lengths of teflon tubing with a 0.5 mm inner diameter. The total volume of the column was 66.2 ml.

The Pharmacia FPLC System (Pharmacia Fine Chemicals, Uppsala, Sweden) consisting of two P-500 pumps, a GP-250 gradient programmer, a Frac-100 fraction collector, one V-7 and two V-8 valves and an UV-1 single path UV monitor was used as high performance liquid chromatograph.

Gel filtration was carried out in 0.15 mol/l sodium chloride containing 3 mmol/l sodium azide pH 7.0. The column connected with a prefilter was operated at room temperature at a flow rate of 0.6 ml/min. Cold samples of HDL containing 1.2-2.2 mg of total protein were injected into the column by means of a 100 or 200 μl sample loop. Effluents were monitored continuously at 280 nm. Fractions of 1 ml were collected and immediately put into ice water. The working pressure of the column system was 4.3 MPa (megapascal), giving a time period of about 120 min for one column volume to pass. It was not found necessary to regenerate the column between runs. However, about 30 ml of eluant was allowed to pass after a completed run of 120 min before a second injection of sample was made.

RESULTS

In order to evaluate the capacity of the high performance gel filtration system used in the present study to subfractionate HDL as a function of particle size, 3 different normal plasmas (A, B and C) were selected representing the most common particle distribution patterns of native HDL we observe in gradient gel electrophoresis. Figure 1 shows the protein-stained gradient gel electrophoretogram of the isolated HDL fractions from the three different plasmas incubated at 0 and 37 C.

Of the HDL fractions isolated from plasma incubated at 0 C, the first (A:0 C) showed two main zones with migration distance centers corresponding to particles with molecular weights of about 300 and 190 kd, respectively. Of these two zones the one matching the largest particles was stained most intensely.

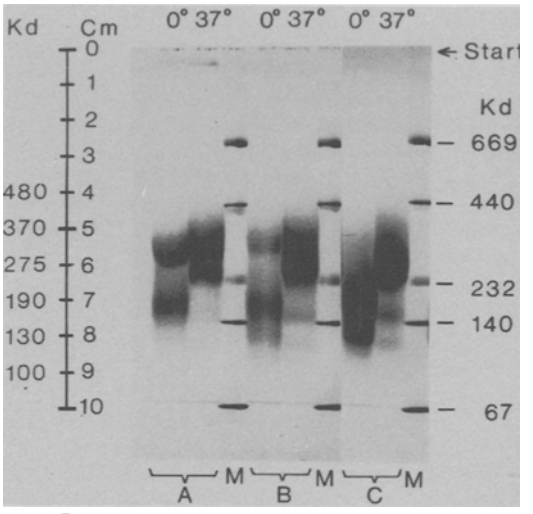

FIG. 1. Electrophoretograms from polyacrylamide gradient gel electrophoresis of HDL isolated from 3 different plasmas; A, B and C incubated at 0 and 37 C for 24 hr. The gel was Pharmacia PAA 4/30. The separated lipoprotein subfractions were stained with Amido black. Cm = migration distance from start in centimeter; Kd = molecular weight in kilodalton; M = marker proteins (kd). Thyroglobulin (669), ferritin (440), catalase (232), lactate dehydrogenase (140), bovine serum albumin (67).

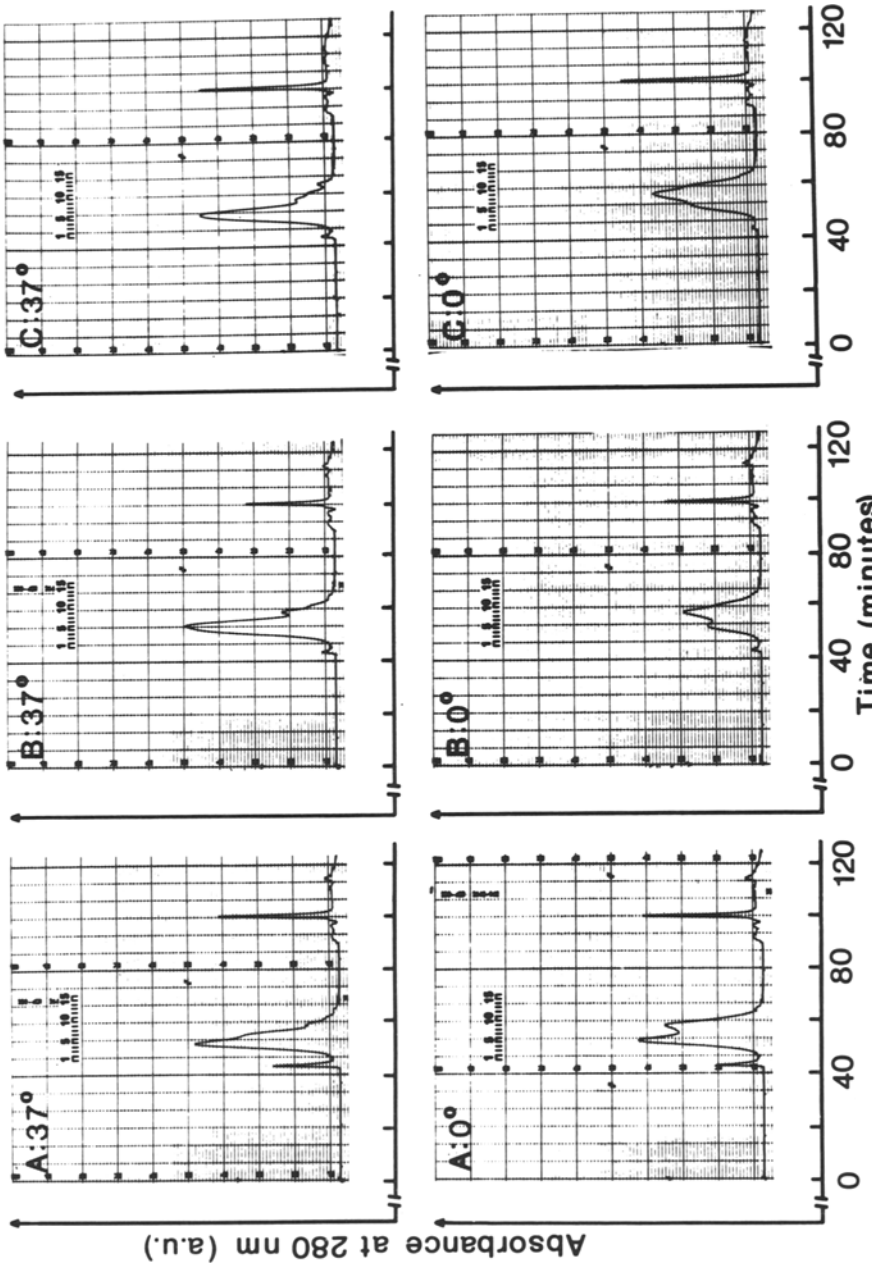

FIG. 2. Photographs of authentic elution curves obtained at high performance gel filtration of HDL isolated from plasma A, B and C incubated at 0 and 37 C for 24 hr. Separation was performed on a 7.5 × 1500 mm TSK-G 3000 SW column using 0.15 mol/l sodium chloride containing 3 mmol/l sodium azide pH 7.00 at a flow rate of 0.6 ml/min and a pressure of 4.3 megapascal (43 kg/cm^2). The inserted scale shows the locations of the 15 collected fractions of 1.0 ml each. a.u. = arbitrary units.

The second plasma produced an HDL (B:0 C) with a similar electrophoretic pattern to A:0 C. In contrast to sample A the zone corresponding to the smallest particles stained most intensely. In addition, a zone appeared agreeing in position with particles of about 130 kd.

In the third HDL sample (C:0 C) the zone representing the high molecular weight particles of about 300 kd was almost non-existent, and the major particle population was seen at a zone corresponding to 190 kd.

Looking at the HDL fractions from the plasmas incubated at 37 C, they all yielded electrophoretic patterns with zones corresponding to particles of about 260 kd. In addition, faintly stained zones representing particle populations of about 160 and 130 kd were present. However, the HDL sample from the first plasma (A:37 C) gave in addition a discrete zone whose center corresponded to particles of about 330 kd. Of the zones in this electrophoretogram, the one agreeing in position to particles of about 330 kd stained most strongly, whereas the corresponding zone in the HDL samples B:37 C and C:37 C was relatively faint and diffuse. Incubation of plasma free from VLDL and LDL was performed under the same conditions at 37 C. The gradient gel electrophoretic pattern as well as the gel filtration profile was identical with those from experiments with whole plasma.

In Figure 2 the elution curves obtained by monitoring the effluents at 280 nm from the gel filtration experiments of HDL isolated from the different plasmas are shown. The resulting subfractionation profiles of the different HDL samples agree very well both in positions of peak maxima and relative peak areas with the particle distribution patterns observed at polyacrylamide gradient gel electrophoresis of the corresponding HDL preparations (Fig. 1). In addition, the peak-resolution of the different particle subfractions of HDL, obtained by high performance gel filtration, equals the resolution obtained with HDL after gradient gel electrophoresis and densitometric scanning of stained zones as originally described by Blanche et al. (3).

The HDL preparations obtained from plasmas incubated at 0 and 37 C were eluted completely from the column in subfractions between 45 and 65 min. The peaks eluting at 100 min at the end of the curves represent low molecular weight compounds originating from plasma. No protein could be detected in this region, and these peaks disappeared completely after dialysis of the samples in Visking 18 tubing against eluant as checked by rechromatography (Fig. 4). The largest of these peaks gives an UV-spectrum identical to that of uric acid with λ_{max}, Ph = 2, 285 nm and λ_{max}, Ph = 7, 292 nm. The small peak eluting in the void volume at 43.3 min (Fig. 2) corresponds to minor contaminants of VLDL, LDL and sinking pre-β lipoproteins which sometimes also could be observed on the electrophoretograms (Fig. 1). In Figure 4 the position of human serum albumin also is marked.

The present gel chromatographic system produced very reproducible elution patterns both regarding absolute positions of peaks and peak heights as demonstrated in Figure 3, show-

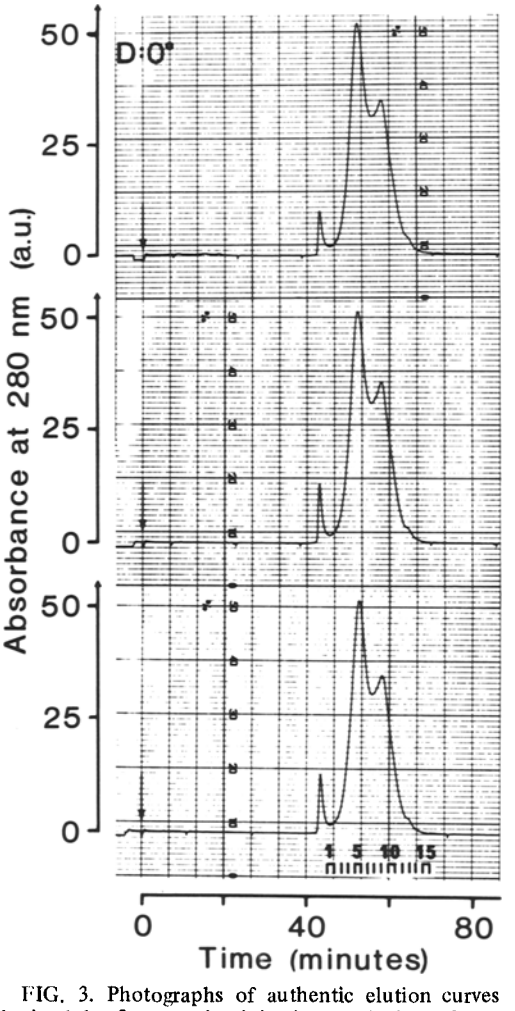

FIG. 3. Photographs of authentic elution curves obtained by 3 successive injections at high performance gel filtration of HDL (D:0 C) isolated from plasma D after incubation at 0 C for 24 hr. Chromatographic parameters same as in Figure 2. Arrows denote state of elution. Sample volume = 200 µl.

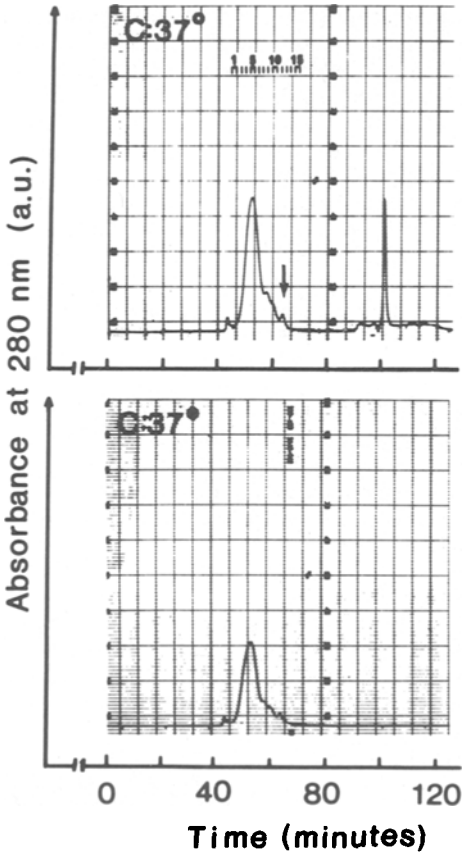

FIG. 4. Photographs of authentic elution curves obtained at high performance gel filtration of HDL isolated from plasma C incubated at 37 C before (above) and after dialysis at 1 C (below) of the HDL sample against 0.15 mol/l sodium chloride containing 3 mmol/l sodium azide pH 7.00. The separation conditions were the same as in Figure 2. The arrow indicates the position of human serum albumin. a.u. = arbitrary units.

ing the elution curves obtained after three successive injections of a native HDL preparation (D:0 C) of a plasma D collected 15 months later from the subject donating plasma to preparation A:0 C and run on the same column as used for the separation of HDL (A:0 C) as presented in Figure 2.

The elution curves in Figure 3 (D:0°) and Figure 2 (A:0°) are obtained using the same chromatographic parameters, and the ruled recorder paper scale allows comparison of the different curves. The concentration values of cholesterol, phospholipids and protein in peak fractions from effluents of three successive fractionations of HDL (D:0 C) as recorded in Figure 3 are presented in Table 1.

When fractions 5 and 8 from the gel filtration experiment with HDL (C:0 C) of plasma C (Fig. 2) were rechromatographed, peaks with maxima at unchanged positions were obtained (Fig. 5).

This gel filtration technique made it possible to recover narrow HDL particle subfractions rapidly (Fig. 5). Analysis of the HDL particles in the different fractions of the column effluents, by polyacrylamide density gradient gel electrophoresis, demonstrated that the HDL preparations could be resolved by gel filtration into particle subfractions with molecular masses which corresponded to particle populations in the original HDL preparation (Fig. 6). In the present study, 15 fractions of 1 ml each were collected in each gel filtration experiment and analyzed for their content of total cholesterol and cholesteryl esters (Fig. 7), apolipoprotein A-I and A-II (Fig. 8) and total phospholipids (Fig. 9). The recovery of HDL after each column separation was better than 90% as calculated for both lipid (total cholesterol) and total protein.

The positions of the peaks of the curves for

TABLE 1

Concentration Values of Lipids and Total Protein in Peak Fractions of Effluents from High Performance Gel Filtration of Native HDL Isolated from Plasma D after Incubation at 0 C for 24 Hours[a]

Fraction number (n = 3)	Total cholesterol (nmol/ml)	Cholesteryl ester (%)	Phospholipids (nmol/ml)	Total protein (μg/ml)
	(mean ± SD)			
5	483 ± 13	69 ± 0	374 ± 43	340 ± 10
6	374 ± 21	70 ± 4	302 ± 28	293 ± 6
9	177 ± 11	81 ± 5	166 ± 14	240 ± 10

[a]The values represent means and standard deviations obtained by analysis of the peak fractions of the effluents from 3 successive injections of HDL preparation D:0 C whose elution curves are shown in Figure 3.

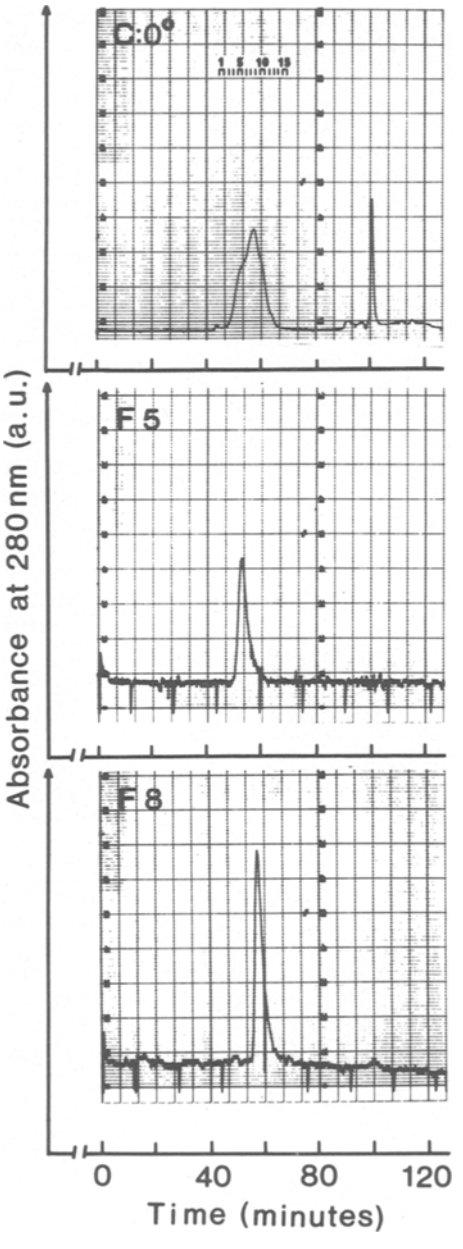

FIG. 5. Photographs of authentic elution curves from high performance gel filtration of HDL isolated from plasma C, incubated at 0 C (above) and rechromatography of fraction 5 (middle) and fraction 8 (below) from this gel filtration experiment. The separation conditions were the same as in Figure 2. a.u. = arbitrary units. The downdirected spikes represent deflections of the printer-pen between the automatic changes of pumps.

total cholesterol and total apolipoprotein A in the different HDL subfractionation experiments agreed very well with the peak positions obtained by monitoring absorbance at 280 nm, which records the total protein concentrations of the HDL subfractions.

A continuing increase in relative cholesteryl ester content could be observed for each of the three HDL samples from plasma incubated at 0 C when going from large to small particles. Thus the HDL population with the smallest particles of about 190 kd had higher cholesteryl ester content relative to total cholesterol than the largest particles of about 300 kd.

After incubation of plasmas at 37 C, the isolated HDL particles eluted from the column at an expected shorter time, confirming the particle enlargement as indicated by the gradient gel electrophoresis technique.

Enlarged HDL particles isolated from plasma incubated at 37 C generally had a higher cholesteryl ester content in relation to total cholesterol than the native HDL particles.

Furthermore, a much smaller difference in relative cholesteryl ester content between small and large particles was present within the enlarged HDL than within the native HDL particle range.

As shown in Figure 8, apolipoprotein A-I in the different HDL particle subfractions obtained by gel filtration of HDL from plasma incubated at 0 and 37 C covaried with the corresponding 280 nm absorbtion curves representing total HDL protein. In addition, Figure 8 shows that apolipoprotein A-II in relation to total apolipoprotein A (A-I + A-II) was fairly constantly distributed among the subfractions of all HDL preparations.

The different plasmas on incubation at 37 C produced enlarged HDL particle-peaks that had essentially the same relative concentration of apolipoprotein A-II as native HDL isolated from the corresponding plasma incubated at 0 C.

When analyzing the distribution of phospholipids between the HDL particle size subfractions of each individual HDL preparation, it was found that in HDL from the plasmas incubated at 0 C the small particles had a higher phospholipid to free cholesterol ratio than the large HDL particles (Fig. 9).

In the enlarged HDL particles originating from plasma A and B incubated at 37 C, however, the ratio of phospholipid to free cholesterol was higher than the ratio for the HDL particles of corresponding size derived from native HDL of plasma stored at 0 C (Fig. 9).

In incubation of enlarged HDL from plasma A which demonstrated the predominant pres-

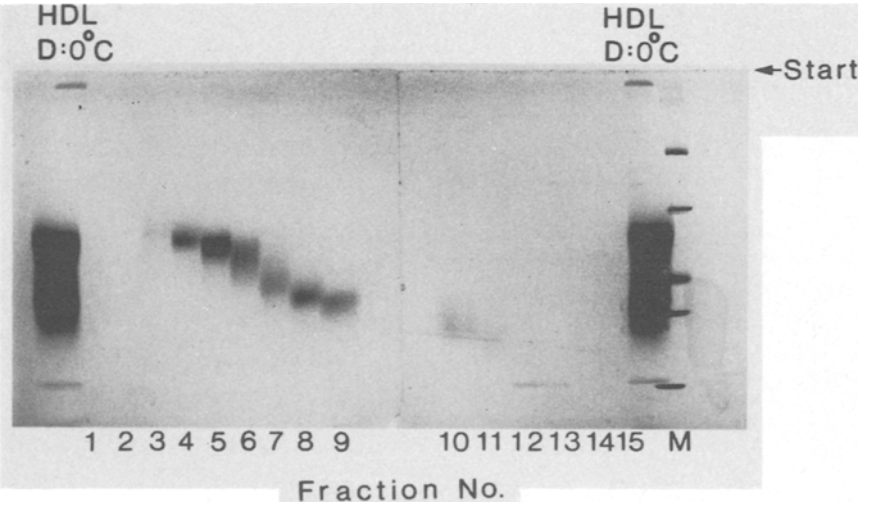

FIG. 6. Electrophoretogram of polyacrylamide gradient gel electrophoresis of isolated native HDL (D:0 C) and 15 HDL particle subfractions obtained by high performance gel filtration as presented and numbered in Figure 3. Injection and fraction volumes were 200 μl and 1 ml respectively (see methods). M = molecular mass markers, same as in Figure 1. Note separation of albumin in fractions 12 and 13.

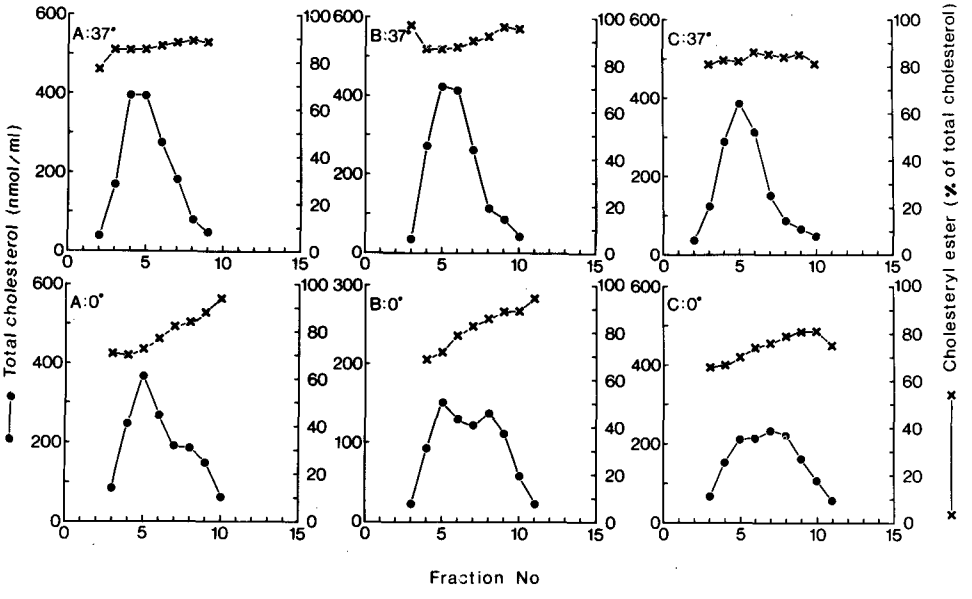

FIG. 7. Concentration of total cholesterol and relative cholesteryl ester content in 1 ml fractions obtained from high performance gel filtration of HDL isolated from plasma A, B and C after incubation of the plasmas at 0 and 37 C for 24 hr. The fraction numbers of the abscissas of the figures refer to the corresponding numbers of the inserted scales in Figure 2.

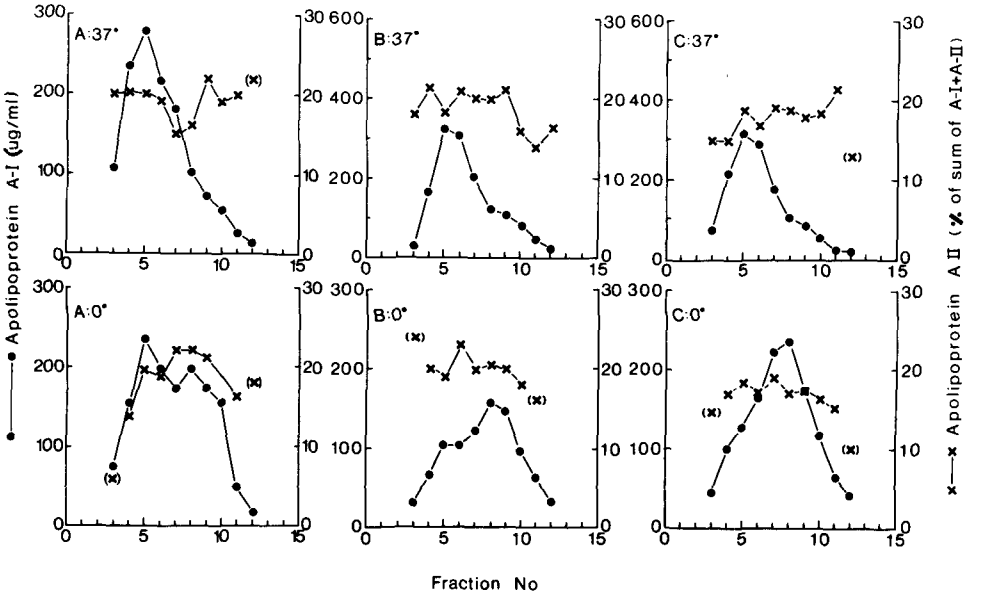

FIG. 8. Concentration of apolipoprotein A-I and relative content of apolipoprotein A-II in the same 1 ml fractions as in Figure 7. The points within brackets at the ends of the curves show the presence of apo A-II, but the values obtained are impaired by a large error of estimation because of the difficulty in measuring accurately the very small immunoprecipitation rockets produced at these very low apolipoprotein concentrations.

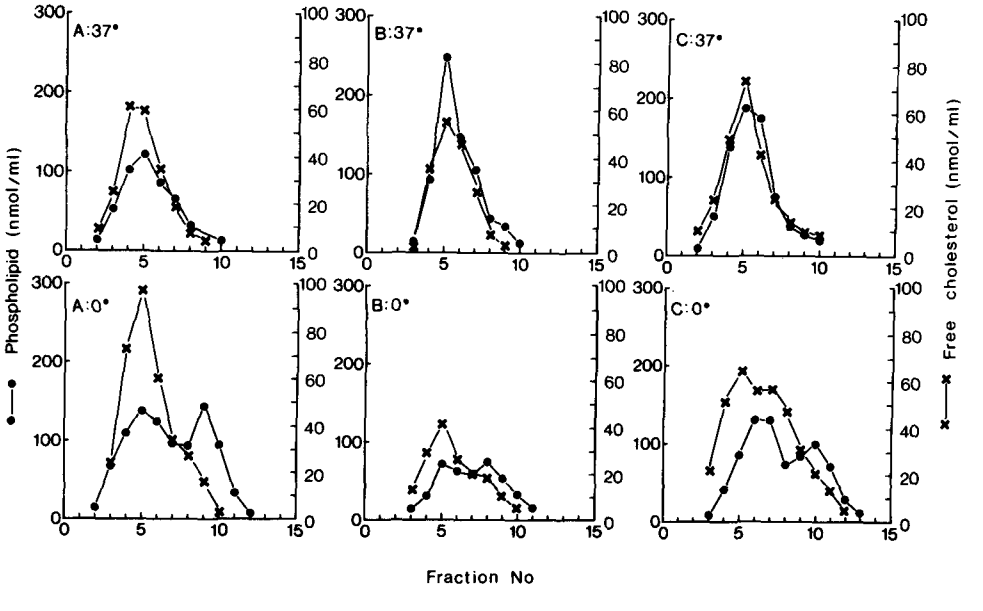

FIG. 9. Concentration of phospholipids and free cholesterol in the same 1 ml fractions as in Figure 7.

ence of a population of large HDL particles already in the native HDL preparation (Fig. 1), the ratio of phospholipid to free cholesterol was markedly lower than the corresponding ratio for the populations of large HDL particles derived from plasma B and C incubated at 37 C (Fig. 9).

DISCUSSION

Using polyacrylamide gradient gel electrophoresis, Blanche et al. (3) have shown that normal HDL after isolation from plasma by preparative ultracentrifugation consists of two major and two minor particle populations which were quantified by densitometric scanning of the stained electrophoretic zones.

Recently we reported (6) that during incubation of plasma at 37 C the HDL lipoproteins increased in particle size as compared to HDL particles in plasma stored at 0 C in accordance with earlier observations (4,5).

We wanted to know the chemical composition of the different particle populations in native and enlarged HDL.

The gradient gel electrophoresis technique, however, is not suitable for complete chemical and immunological characterization of the HDL particle fractions trapped in the gel matrix.

A method for quantification of cholesterol in human serum HDL after fractionation of whole serum by means of high performance gel filtration utilizing coupled TSK-G 5000 PW and G 3000 SW columns has, however, been described (9). Using three coupled TSK-G 3000 SW columns of a total length of 150 cm, we have achieved a separation of different particle size populations of HDL to match the subfractionation obtained with the polyacrylamide gradient gel electrophoresis technique (3).

In the present study HDL preparations isolated by ultracentrifugation from different plasmas incubated at 0 and 37 C were separated in particle subfractions within 65 min (Fig. 2). The small peak appearing in the void volume at 43.3 min corresponded to contaminants of apolipoprotein B containing lipoproteins. Only traces of apolipoprotein A-I could be detected in this fraction, demonstrating that the presence of large HDL aggregates, like stacked discs, seems negligible.

The recoveries from the columns were essentially quantitative, and no contamination of the column with material from HDL could be detected at a check of the column after injection of 20 HDL samples (producer's recommendation). Successive injections of an HDL preparation followed by quantitative analysis of lipids and protein of peak fractions revealed a good reproducibility over a period of 15 mo both regarding absolute positions of peaks and chemical compositions of the fractions (Fig. 2 and 3, Table 1).

Rechromatography of an HDL sample from plasma gave an unchanged elution pattern (Fig. 4). When fractions from gel filtration experiments with native HDL were rechromatographed, the positions of the peak maxima of the HDL fractions coincided exactly with the corresponding peak maxima obtained by gel filtration of the native HDL preparation (Fig. 5). These findings in combination with the symmetric appearance of the peaks in the rechromatographic experiments and the results from the polyacrylamide gradient gel electrophoresis of fractions obtained after gel filtration of an HDL preparation (D:0 C) (Fig. 6) indicate that the HDL particles do not decay or change in composition during the gel filtration procedure.

The peak-resolution obtained with the present high performance gel filtration was found to be equal to that we obtain with the gradient electrophoresis technique (3), and the migration distances of the zones representing different HDL particle populations in gradient gel electrophoresis corresponded to expected elution time periods in the gel filtration experiments. Also, the relative magnitudes of the areas associated with the peaks were similar in the two methods.

The different particle size subfractions obtained after gel filtration of HDL isolated from plasma incubated at 0 and 37 C were characterized by determination of their content of total and esterified cholesterol, apolipoproteins A-I and A-II and phospholipids (Figs. 7-9). Apolipoproteins C and E were not considered in the present study. However, preliminary experiments did not indicate loss of apolipoprotein C or E during the gel filtration procedure as analyzed by enzymeimmunoassays (21). (Results not reported here.)

The results from the compositional analysis of the different HDL subfractions indicated that enlarged HDL particles from plasma incubated at 37 C had a higher relative cholesteryl ester content than particles of similar size from plasma incubated at 0 C.

The content of phospholipids in total enlarged HDL isolated from plasma A, B and C after incubation at 37 C was found to be reduced 20 to 30% compared to total native HDL. However, the content of total cholesterol in the corresponding HDL pairs was the same in native and enlarged HDL particles.

The present results suggest that the incubation enlargement of HDL particles is linked to the esterification of free cholesterol by lecithin:

cholesterol:acyl transferase (LCAT EC 2.3.1.43), with the formation of lysolecithin which might leave the HDL particle. However, the enzyme activity per se does not explicitly seem to be primarily responsible for the enlargement of normal native HDL particles, as it has been demonstrated that an enlargement process might also occur in the presence of an LCAT inhibitor (4,5). A role of LCAT in the HDL particle enlarging process cannot be excluded, however, solely on the basis of inhibition of its catalytic activity by a specific blocking of sulfhydryl groups of the active center of the enzyme. The polypeptide backbone of LCAT having sequences with specific affinity for the HDL surface lipids and apolipoproteins might still induce or aid fusion of HDL particles.

The difference in phospholipid concentration profiles between native and enlarged HDL preparations also indicates that the small cholesteryl ester rich HDL particles are preferentially enlarged. This enlargement seems to be independent of the presence of VLDL and LDL, as the removal of these lipoproteins before incubation did not affect the enlargement as checked by electrophoresis and high performance gel filtration.

The concentration profiles of the elution curves for total cholesterol, apolipoproteins A-I and A-II of the different HDL preparations agreed very well with the curves obtained by monitoring the eluants at 280 nm.

The almost constant relative occurrence of apolipoprotein A-II throughout the different native and enlarged HDL subfractions indicates a close association between these two apolipoproteins.

The present results demonstrate that high performance gel filtration is a rapid and reproducible means for subfractionation of HDL as a function of particle size allowing quantitative determinations of lipid and protein components of the HDL particles.

ACKNOWLEDGMENT

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COMMUNICATIONS

The Lipid Composition of the Spleen and Intestinal and Popliteal Lymph Nodes in the Sheep

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ABSTRACT

Lipids were isolated from the spleen, intestinal lymph nodes and popliteal lymph nodes, tissues of potential importance in the metabolism of lymph or lymphocytes, of the sheep. The most abundant lipid classes were the triacylglycerols, free cholesterol and phospholipids, especially phosphatidylcholine, phosphatidylethanolamine and sphingomyelin. In each lipid class, the amounts of the essential fatty acids were lower than in the corresponding lipids of lymph or plasma. The triacylglycerols of each tissue resembled those of adipose tissue in structure, rather than those of lymph or plasma. Also, the structures of the phosphatidylcholines were distinctive. The results are discussed in terms of the metabolism of lipids in lymph. It does not appear probable that lymphoid tissue can supply appreciable amounts of lipid to lymph.
Lipids 20:389-392, 1985.

INTRODUCTION

In a recent study of the lipid composition of the lipoproteins of intestinal and popliteal lymph in the sheep, it was observed that the content of the essential fatty acids, especially linoleic acid, was particularly high for a ruminant tissue (1,2). The origin of these components is obscure because biohydrogenation in the rumen limits the supply from the diet (3), and very little can be recycled via the bile (4). The most probable source is the plasma lipids, which could be catabolized directly by the intestinal cells or which could be transferred to lymph by capillary filtration via the lymph nodes or by exchange from other tissues involved in the metabolism of lymph constituents, e.g., the spleen, which contains the largest accumulation of lymphoid tissue in the body and serves as one source of lymphocytes. In order to assess the potential of the last two routes to the supply of essential fatty acids to lymph in the sheep, the lipid compositions of the spleen and the intestinal and popliteal lymph nodes have been determined.

TABLE 1

Lipid Content, Neutral Lipid Class Composition and Phospholipid Class Composition of Spleen, Intestinal Lymph Nodes and Popliteal Lymph Nodes of Sheep

	Spleen	Intestinal lymph node	Popliteal lymph node
Lipid content			
% Lipid (of wet weight)	1.8 ± 0.32	2.0 ± 0.52	7.7 ± 1.31
% Lipid (of dry matter)	9.9 ± 2.82	12.1 ± 2.47	37.6 ± 10.09
Neutral lipids			
Cholesterol esters	0.3 ± 0.3	1.0 ± 0.5	1 ± 1
Triacylglycerols	28 ± 16	57 ± 18	82 ± 3
Free fatty acids	1 ± 0.7	1 ± 0.6	–
Cholesterol	30 ± 10	12 ± 5	5 ± 1
Diacylglycerols	1 ± 0.4	1 ± 0.4	–
Phospholipids (total)	40 ± 5	29 ± 12	12 ± 2
Phospholipids			
Cardiolipin	3 ± 2	6 ± 4	3 ± 1
Phosphatidylethanolamine	25 ± 3	21 ± 4	21 ± 5
Phosphatidylethanolamine-plasmalogen	9 ± 3	6 ± 1	8 ± 1
Phosphatidylserine	5 ± 3	6 ± 2	5 ± 2
Phosphatidylserine	5 ± 3	3 ± 1	4 ± 2
Phosphatidylcholine	47 ± 6	52 ± 5	48 ± 4
Sphingomyelin	8 ± 2	6 ± 2	12 ± 1

Results are expressed as means ± S.D. (n=4).
Neutral lipid and phospholipid class compositions are given as wt % of total lipids.

TABLE 2

Positional Distributions of Fatty Acids (Mol % of the Total) in the Triacyl-*sn*-glycerols of Spleen, Intestinal Lymph Nodes and Popliteal Lymph Nodes of Sheep

Fatty acid	Spleen			Intestinal lymph node			Popliteal lymph node		
	sn-1	*sn*-2	*sn*-3	*sn*-1	*sn*-2	*sn*-3	*sn*-1	*sn*-2	*sn*-3
14:0	1.4 ± 0.82	3.3 ± 0.93	4.5 ± 1.47	2.1 ± 1.10	3.5 ± 0.45	7.1 ± 1.68	2.2 ± 1.0	3.1 ± 1.1	6.7 ± 1.50
15:0	0.8 ± 0.31	0.5 ± 0.22	0.3 ± 0.2	0.9 ± 0.24	0.9 ± 0.17	1.7 ± 0.21	0.8 ± 0.3	0.4 ± 0.9	1.1 ± 0.37
16:0	35.5 ± 3.99	17.2 ± 1.86	17.1 ± 4.01	37.2 ± 4.41	22.0 ± 2.81	23.7 ± 3.38	47.0 ± 4.15	15.8 ± 2.56	16.8 ± 2.92
16:1	1.5 ± 0.96	1.7 ± 0.55	0.8 ± 0.29	0.7 ± 0.20	1.6 ± 0.22	1.7 ± 0.39	0.8 ± 0.32	1.8 ± 0.75	2.8 ± 0.31
17br	2.0 ± 0.69	0.9 ± 0.39	1.6 ± 0.35	2.2 ± 0.41	0.9 ± 0.61	3.6 ± 1.43	2.2 ± 0.72	0.6 ± 0.07	2.1 ± 0.61
17:0	1.7 ± 0.75	0.6 ± 0.17	1.8 ± 0.46	2.0 ± 0.41	0.6 ± 0.19	1.9 ± 0.26	2.4 ± 0.26	0.6 ± 0.10	0.8 ± 0.40
18:0	42.7 ± 6.9	12.1 ± 4.52	26.0 ± 4.18	44.5 ± 4.20	13.7 ± 2.91	31.6 ± 3.59	30.3 ± 3.31	13.2 ± 2.07	23.6 ± 3.85
18:1	13.9 ± 4.05	57.5 ± 2.18	45.2 ± 6.55	10.3 ± 2.74	51.3 ± 4.64	28.0 ± 3.99	14.1 ± 1.34	60.4 ± 3.68	44.2 ± 2.99
18:2	0.3 ± 0.34	5.3 ± 1.43	2.0 ± 0.61	0.2 ± 0.11	5.3 ± 0.32	1.0 ± 0.28	0.2 ± 0.13	4.0 ± 0.60	1.5 ± 0.45

Means (± S.D.) of results from 4 animals.

EXPERIMENTAL METHODS

Tissues

The animals used were 5-year-old wethers of the Clun Forest breed, between 30 and 50 kg in weight. They were fed a hay-concentrate diet twice daily with water available ad libitum. The spleen and popliteal and intestinal lymph nodes were dissected out after the animals had been killed by a captive-bolt pistol. Any adhering adipose tissue was carefully removed.

Analytical Methods

Lipids were extracted from the tissues, lipid classes separated and fatty acid compositions determined as described previously (1). Methods for the structural analyses of phosphatidylcholines and triacylglycerols also have been described elsewhere (2,5,6).

RESULTS AND DISCUSSION

The lipid content, neutral lipid class composition and phospholipid class composition of the spleen, intestinal lymph nodes and popliteal lymph nodes of the sheep are listed in Table 1. The popliteal lymph nodes contained more lipid than the other tissues, both on a wet weight and a dry matter basis. No other data is available on the lipid contents of these tissues, but bovine spleen contained 5.06% of the wet weight of lipid (7). Triacylglycerols, free cholesterol and phospholipids were the main lipid classes in each of these tissues, with triacylglycerols dominant in the lymph nodes. The phospholipid compositions of the three tissues (8) were broadly similar to each other and to those of most sheep tissues, with phosphatidylcholine comprising about half the total and phosphatidylethanolamine and sphingomyelin making up most of the remainder. Smaller amounts of phosphatidylethanolamine were found in a previous analysis of sheep spleen phospholipids (9), but the results reported here were similar to those recorded for bovine spleen (7,10,11).

The fatty acid compositions of the main lipid classes of each of the tissues were determined (data not shown). In the triacylglycerols, mainly C_{16} and C_{18} fatty acids were found with relatively little linoleic acid; the intestinal lymph node contained more saturated fatty acids than the other tissues. All resembled the internal depot fats in composition (8). The phosphatidylethanolamines contained the highest proportion of polyunsaturated fatty acids, as might be expected, with arachidonic acid the most abundant component, together with appreciable amounts of C_{22} polyunsaturated fatty acids, but relatively little linoleic acid. The phosphatidylcholines from each tissue also were similar

in composition and contained somewhat less of the C_{20} and C_{22} polyunsaturated fatty acids but more linoleic acid than the phosphatidylethanolamines.

Stereospecific analyses were performed to determine the distributions of fatty acids in positions *sn*-1, *sn*-2 and *sn*-3 of the triacylglycerols; the results are listed in Table 2. Although the triacylglycerols were found to be highly asymmetric, with saturated fatty acids in greatest concentration in position *sn*-1, unsaturated fatty acids in greatest abundance in position *sn*-2 and longer-chain fatty acids in position *sn*-3, there were few differences between tissues. The triacylglycerols resembled those of adipose tissue (12) rather than of plasma (2,12) and lymph (2,13) (or adrenals [5]), in which palmitic acid is concentrated in position *sn*-2.

The positional distributions of fatty acids in the phosphatidylcholines of each of the tissues also were determined, and the results are shown in Table 3. In the phosphatidylcholine of spleen, position *sn*-1 contained mainly saturated fatty acids; the polyunsaturated fatty acids were concentrated in position *sn*-2. However, there was almost as much palmitic acid in position *sn*-2 as in position *sn*-1. The phosphatidylcholines of the intestinal and popliteal lymph nodes were somewhat similar, although there was proportionately less palmitic acid in position *sn*-2. Therefore, they resembled the structures of phosphatidylcholine in ruminant brain (14,15), thymus (also a lymphoid tissue) (16), or lung (17), rather than those of lymph (2,13), plasma (2,17,18) or liver (12,20,21), in which there is very little palmitic acid in position *sn*-2.

Because of the uncommon structure of the phosphatidylcholines in the spleen especially, the proportions of the various molecular species were determined by high-temperature gas-liquid chromatography and silver-ion thin-layer chromatography following conversion to the diacylglycerol acetate (6). As might be anticipated, relatively high proportions of di-saturated species (29% of the total) were found in comparison to most of the natural phosphatidylcholines which have been analyzed (22).

There was little compositional resemblance between the lipids of spleen and the lymph nodes, and the lipid structures were very different. The results of a purely analytical study of this kind rarely can be used to prove a biochemical point. However, they do appear to indicate that other lymphoid tissues are unlikely to transfer appreciable amounts of linoleic acid or of intact lipids to lymph. A more fruitful biochemical approach might be to study the catabolism of plasma high-density lipoproteins in sheep intestinal tissue, a process which appears to occur in the rat (23,24).

TABLE 3

Positional Distributions of Fatty Acids in the Phosphatidylcholines of Spleen, Intestinal Lymph Nodes and Popliteal Lymph Nodes of Sheep

	Spleen[a]		Intestinal lymph node[a]		Popliteal lymph node[b]	
Fatty acid	*sn*-1	*sn*-2	*sn*-1	*sn*-2	*sn*-1	*sn*-2
14:0	1.4 ± 0.39	1.1 ± 0.40	0.7 ± 0.17	0.7 ± 0.33	0.9	0.2
15:0	1.8 ± 0.42	1.5 ± 0.04	1.5 ± 0.32	1.2 ± 0.50	1.1	0.4
16:0	43.9 ± 3.80	34.7 ± 1.42	36.3 ± 2.66	25.9 ± 2.53	33.3	16.8
16:1	1.6 ± 0.52	2.5 ± 0.20	1.6 ± 0.20	2.1 ± 0.04	1.9	1.9
17br[c]	3.4 ± 0.55	2.9 ± 0.40	3.1 ± 0.40	1.8 ± 0.42	3.0	1.8
17:0	2.0 ± 0.25	0.5 ± 0.04	2.2 ± 0.40	0.3 ± 0.02	2.0	0.4
18:0	27.2 ± 3.67	2.3 ± 0.72	31.9 ± 0.91	1.9 ± 0.53	34.4	3.0
18:1	15.9 ± 2.12	21.0 ± 1.30	18.0 ± 0.31	14.8 ± 2.70	21.3	21.3
18:2	1.5 ± 0.33	17.0 ± 0.88	2.5 ± 0.39	17.1 ± 0.96	2.1	16.5
20:3(n-6)	0.1 ± 0.08	0.9 ± 0.12	–	1.1 ± 0.08	–	1.7
20:3(n-3)	0.1 ± 0.09	0.8 ± 0.20	–	1.9 ± 0.26	–	2.0
20:4(n-6)	0.1 ± 0.12	11.2 ± 1.54	–	25.9 ± 3.71	–	25.7
22:5(n-3)	–	1.3 ± 0.35	–	2.9 ± 0.56	–	4.9
22:6(n-3)	–	0.8 ± 0.39	–	1.1 ± 0.35	–	1.5

[a]Mean (± S.D.) of results from 3 animals.

[b]Analysis of pooled sample from 4 animals.

[c]br = A mixture of iso- and anteiso-branched components.

Fatty acids are given as mol % of the total.

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The Monoenoic Fatty Acid Composition of a Marine Species of *Desulfobulbus* Grown on Lactate

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ABSTRACT

The proportions of isomeric monoenoic fatty acids from cell lipids of a marine, anaerobic, sulphate-reducing bacterium *Desulfobulbus*, grown on lactate, were determined by pyrrolidide derivatization and GC-MS analysis. The predominant fatty acid was C17:1, Δ11 with much smaller proportions of C16:1, Δ11 and C18:1, Δ11 and small (<5%) yet significant proportions of C15:1, Δ11; C16:1, Δ9; C17:1, Δ9 and C18:1, Δ9. If the spectrum of isomers obtained from this organism were synthesized entirely by the anaerobic pathway, it would indicate a wider chain-length specificity for the rate-limiting β-hydroxy acyl dehydrase than hitherto has been reported. The high proportion of odd-numbered fatty acids reflected the chain initiation by propionate derived from lactate. The possibility of monoene synthesis by an anaerobic Δ9-desaturase mechanism is discussed.
Lipids 20:393-397, 1985.

INTRODUCTION

Both aerobic and anaerobic pathways have been described for the biosynthesis of long-chain mono-unsaturated fatty acids in various microorganisms (1). The distribution of the double-bond in the positional isomers of the cellular monoenoic fatty acids of microorganisms is considered to be a reflection of the operation of a particular biosynthetic pathway (2).

The aerobic desaturase reaction introduces the double bond into a preformed long-chain, saturated fatty acid usually between the Δ9-10 carbons of the chain (3,4). This reaction has an obligatory requirement for NADH/NADPH as electron donors, for a short, non-phosphorylating electron transport chain with the terminal desaturase enzyme and for molecular oxygen as the terminal electron acceptor (1). The products of this desaturase mechanism are Δ9-10 monoenoic fatty acids of various chain length; often chain elongation of the monoenoic acid occurs subsequent to desaturation (c.f. 2).

In contrast, the anaerobic mechanism for monoenoic fatty acid synthesis involves the chain elongation of a Δ3 medium chain monoenoic fatty acid which is produced by the action of β-hydroxy decanoyl thioester dehydrase on a β-hydroxy fatty acid substrate. This enzyme has a narrow chain-length specificity, and its preferred substrate is the C10 chain-length β-hydroxy fatty acid; little activity is shown toward C_8 or C_{12} fatty acids (1). The products of the elongation of such a C10, Δ3 monoenoic acid by two or four carbons are C16:1, Δ9 and C18:1, Δ11 and both isomers will have the double bond in the ω7 position when counting from the methyl end of the carbon chain (c.f. 1). This anaerobic reaction does not require any electron transfer systems or electron acceptors.

A previous publication described the cellular fatty acids of the anaerobic, sulphate-reducing bacteria *Desulfobacter* sp., *Desulfobulbus* sp. and *Desulfovibrio desulfuricans* (5). The present report gives a more detailed study of the monoenoic fatty acids of *Desulfobulbus* sp. A re-assignment of some double-bond positions reported in the original paper has been made on the basis of detailed GC-MS data obtained for the pyrrolidide derivatives of the monoenoic fatty acids (6). The results show that C17:1, Δ11 is the most abundant fatty acid with a range of Δ9 and Δ11 isomers in the chain lengths C15 to C18. Two possible mechanisms for the biosynthesis of these double-bond isomers are considered and discussed.

METHODS

Culture Conditions

Desulfobulbus sp. strain 3 pr 10 isolated from the anerobic mud flat of the Jadebusen (North Sea) and supplied by F. Widdel, University of Konstanz, West Germany (7) was used in this experiment. The organism was grown on a marine medium with lactate as growth substrate as described by Taylor and Parkes (5).

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Extraction and Isolation of Fatty Acids

The bacterial culture was harvested by centrifugation at 34,000 g for 20 min. The fatty acids were extracted and isolated as described by Taylor and Parkes (5). The monoenoic fatty acid methyl esters were further separated by argentation TLC and converted to their pyrrolidide derivatives as described by Anderson and Holman (6). The monoenoic fatty acids were analyzed as their methyl esters by GLC using a Carlo Erba FTV 4160 gas chromatograph fitted with a 25 m OV-1 glass capillary column programmed from 70-290 C at 4 C/min^{-1}. Mass spectral data were obtained from the pyrrolidide derivatives using a VG16F capillary GC-MS operated at 70 eV and fitted with a 25 m Sil 5 column operated isothermally at 220 C.

RESULTS AND DISCUSSION

The chromatographic analysis of the monoenoic fatty acids from total cell lipids of *Desulfobulbus* sp. is presented in Figure 1. Peak identification was based on mass spectral data of the corresponding pyrrolidide derivatives. The mass spectra obtained for N-hexadec-9-enoyl-pyrrolidide (Δ9-C16:1) and the isomeric N-hexadec-11-enoylpyrrolidide (Δ11-C16:1) are shown in Figure 2. In the pyrrolidide derivatives, unlike their corresponding methyl esters, the amide group has a charge stabilization effect upon the fatty acid moiety which reduces the tendency of the double bond to migrate under electron impact (8). This results in a relatively simple cleavage pattern in the high mass region with fragments of 14 atomic mass units derived from cleavage at each C-C bond in the fatty acid chain. Double-bond positions are simply identified by fragments with an interval of 12 atomic mass units instead of the regular 14 (c.f. Fig. 2).

The percentage composition of monoenoic fatty acids in the total lipids extracted from *Desulfobulbus* grown on lactate is given in Table 1, together with their respective equivalent chain lengths determined from the capillary GC data and the characteristic mass fragments of the pyrrolidide derivatives. The most abundant fatty acid in this organism is the C17:1, Δ11 isomer which comprises 59% of the total fatty acids, followed by the C16:1, Δ11 and the C18:1, Δ11 isomers, which comprise 16% and 10% of the total, respectively (Table 1). All the monoenoic fatty acids found in *Desulfobulbus* could be derived by the 'normal' anaerobic mechanism of synthesis (1). This would involve the activity of a dehydrase and the formation from their β-hydroxy fatty acid precursors of a series of Δ3-monoenoic fatty acids of chain length C7-C12 which subsequently are elongated to various extents to yield the end products found (Table 2). The predominant C17:1, Δ11 fatty acid is therefore derived from the C9:1, Δ3 precursor and the

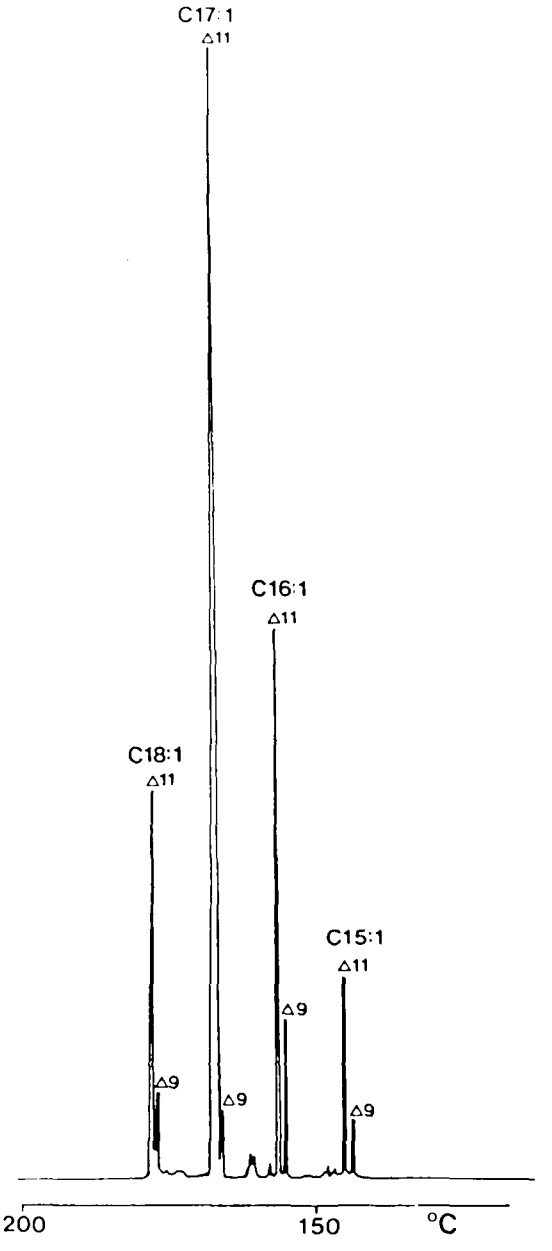

FIG. 1. Gas-liquid chromatogram of monoenoic fatty acid methyl esters from *Desulfobulbus* sp. on a 25 m glass WCOT column coated with OV-1.

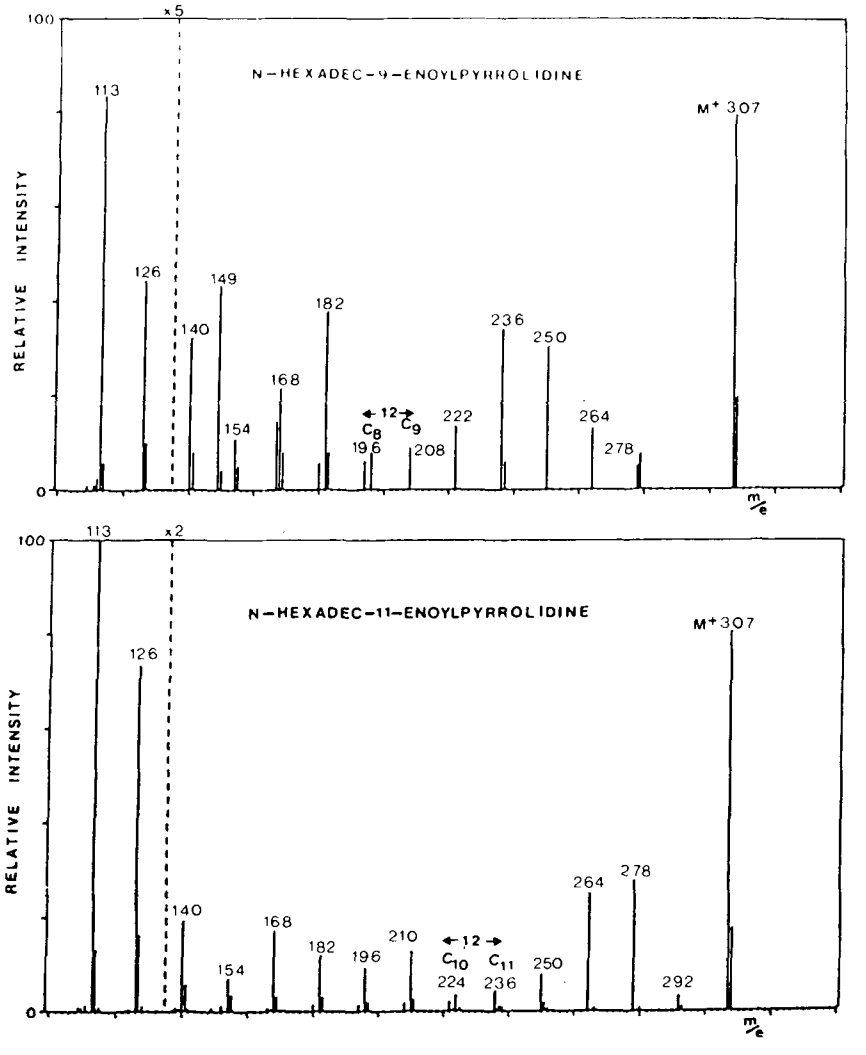

FIG. 2. Mass spectra of N-hexadec-9-enoylpyrrolidide C16:1, Δ9 and N-hexadec-11-enoylpyrrolidide C16:1, Δ11.

TABLE 1

The Distribution of Monoenoic Fatty Acids of *Desulfobulbus* sp.

Double-bond position	ECL OV-1	%	Characteristic mass fragments of pyrrolidide derivatives ($\frac{m}{e}$)
C15:1 ω6, Δ9	14.88	1	196, 208, 293 (M^+)
C15:1 ω4, Δ11	14.92	4	224, 236, 293 (M^+)
C16:1 ω7, Δ9	15.85	3	196, 208, 307 (M^+)
C16:1 ω5, Δ11	15.90	16	224, 236, 307 (M^+)
C17:1 ω8, Δ9	16.88	2	196, 208, 321 (M^+)
C17:1 ω6, Δ11	16.95	59	224, 236, 321 (M^+)
C18:1 ω9, Δ9	17.75	2	196, 208, 335 (M^+)
C18:1 ω7, Δ11	17.80	10	224, 236, 335 (M^+)

TABLE 2

Possible Pathways of Lipid Biosynthesis to Account for the Distribution of Monoenoic Fatty Acids of *Desulfobulbus* sp.

Anaerobic pathway of lipid biosynthesis			% Product found in the cell fatty acids of *Desulfobulbus* sp.	Aerobic pathway of lipid biosynthesis			
Precursor	Elongation	Product		Product	Elongation	Desaturation	Saturated precursor
C7:1 ω4, Δ3	+ $4C_2$	C15:1 ω4, Δ11	4	C15:1 ω4, Δ11	+ C_2	C13:1 ω4, Δ9	C13:0
C8:1 ω5, Δ3	+ $4C_2$	C16:1 ω5, Δ11	16	C16:1 ω5, Δ11	+ C_2	C14:1 ω5, Δ9	C14:0
C9:1 ω6, Δ3	+ $3C_2$	C15:1 ω6, Δ9	1	C15:1 ω6, Δ9	none	C15:1 ω6, Δ9	C15:0
C9:1 ω6, Δ3	+ $4C_2$	C17:1 ω6, Δ11	59	C17:1 ω6, Δ11	+ C_2	C15:1 ω6, Δ9	C15:0
C10:1 ω7, Δ3	+ $3C_2$	C16:1 ω7, Δ9	3	C16:1 ω7, Δ9	none	C16:1 ω7, Δ9	C16:0
C10:1 ω7, Δ3	+ $4C_2$	C18:1 ω7, Δ11	10	C18:1 ω7, Δ11	+ C_2	C16:1 ω7, Δ9	C16:0
C11:1 ω8, Δ3	+ $3C_2$	C17:1 ω8, Δ9	2	C17:1 ω8, Δ9	none	C17:1 ω8, Δ9	C17:0
C12:1 ω9, Δ3	+ $3C_2$	C18:1 ω9, Δ9	2	C18:1 ω9, Δ9	none	C18:1 ω9, Δ9	C18:0

C16:1, Δ11 and C18:1, Δ11 acids from the C8:1, Δ3 and C10:1, Δ3 precursors respectively (Table 2). Bloch (1) showed that, in a variety of anaerobic microorganisms, the dehydrase enzyme has a very high specificity for the C10 β-hydroxy fatty acid and that the major monoenoic acids found in their lipids are derived from the C10:1, Δ3 precursor with only trace amounts of fatty acids derived from other Δ3 monoenoic precursors. The spectrum of long-chain monoenoic fatty acid isomers present in the lipids of *Desulfobulbus* therefore suggests that the dehydrase in this organism has a much broader substrate specificity than that reported for this enzyme in other anaerobic microorganisms (c.f. 1).

The predominance of the odd-carbon number C17:1, Δ11 fatty acid in *Desulfobulbus* probably is a reflection of the enhanced availability of C3 moieties for chain initiation processes in fatty acid synthesis when the organism is grown on lactate or propionate. When the organism is grown on H_2/CO_2 the even carbon number isomers of monoenoic fatty acids predominate, but their proportional distribution still indicates the same breadth of specificity of the dehydrase enzyme (5).

Because only Δ9 and Δ11 monoenoic fatty acid isomers occur in *Desulfobulbus* it is tempting to postulate that these may be derived by direct Δ9-desaturation of a series of saturated fatty acid precursors of chain-length C13-C18 with (Δ11) or without (Δ9) subsequent chain elongation by C_2 units (Table 2). Such a mechanism would be analogous to the aerobic Δ9-desaturase reaction (1), but without a similar requirement for molecular oxygen as the terminal electron acceptor. The possible existence of such an anaerobic Δ9-desaturase was first indicated by the work of Shapiro and Wertheimer (9), using rat adipose tissue, and by Bloomfield and Bloch (10), who showed that the rate of anaerobic Δ9-desaturation in yeast was about 10% of that found under aerobic conditions. Furthermore, Kemp et al. (11) also have reported that the unsaturated fatty acids of the anaerobic ruminal phycomycete *Piromonas communis* were the product of a Δ9-desaturase which uses stearic acid as substrate and does not require molecular oxygen for activity. Preliminary evidence from our laboratories has indicated that *Desulfobulbus* also is capable of desaturating palmitic acid under anaerobic conditions, albeit at a low rate, thus lending further support to the existence of an anaerobic desaturase system. Work is continuing to establish the relative importance of this anaerobic desaturase pathway to the overall biosynthesis of monoenoic fatty acids in the

organism and to ascertain the type of electron acceptor and hence the possible mechanism of the reaction.

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Letter to the Editor

Sir:

Separation of polar from non-polar lipids of total lipid extracts is a useful first step in the purification and analysis of lipids. The procedure recently reported by Juaneda and Rocquelin (1) offers a rapid a convenient method for the separation of phospholipids from neutral lipids of rat heart. Earlier, we reported a very similar procedure (2,3) for the separation of phospholipids and neutral lipids from extracts of human milk using the same commercially available, prepacked and disposable silica gel Sep-Pak cartridges. Yet, slightly different solvent systems were employed. We used hexane/diethyl ether (1:1) to elute the neutral lipids compared to chloroform, which Juaneda and Rocquelin (1) used and which they found inadequate to completely resolve monoglycerides from phospholipids. To elute the phospholipids, we used methanol, followed by chloroform/methanol/water (3:5:2). For the past five years, we have successfully utilized the commercial Sep-Pak cartridges for the routine separation of neutral lipids from phospholipids of tissue extracts or of the lipids of milk from human and animal sources.

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Decrease in Alkaline Triglyceride Lipase in Primary Cultured Hepatocytes from Mice with Sarcoma 180

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ABSTRACT

Primary cultured hepatocytes from normal mice and mice with Sarcoma 180 were characterized. The viability of freshly isolated hepatocytes from both sources was over 90% and the cells had a relatively stable population of DNA for a minimum of three days. After incubation with (^{3}H)leucine, the syntheses and secretions of (^{3}H)labeled trichloracetic acid-insoluble materials by hepatocytes from both normal and tumor-bearing mice increased similarly. However, the alkaline triglyceride lipase activity of a homogenate of freshly isolated hepatocytes from tumor-bearing mice was one-third that of cells from normal mice. The activity of hepatocytes from tumor-bearing mice increased less during culture than did the activity of cells isolated from normal mice.
Lipids 20:399-403, 1985.

INTRODUCTION

Lipid metabolism in humans and animals often changes significantly during growth of tumors. This change could be due to disturbance of the processes for removing lipids from the blood, hypersecretion of lipids into the blood, or both.

At least two lipolytic enzymes are known to be involved in the metabolism of lipoproteins present in the blood. One is hepatic triglyceride lipase (hepatic TGL), which has a pH optimum for triglyceride hydrolysis of 8-9, is relatively resistant to a high concentration of NaCl and does not require an apolipoprotein cofactor (1-3). Hepatic TGL is known to originate in the liver (2) and to be released by heparin into a liver perfusate or plasma (1-5). The other enzyme is lipoprotein lipase (LPL), which has an alkaline pH optimum, is inhibited by a high concentration of NaCl and requires an apolipoprotein cofactor for maximal activity (6-10).

Previously we reported that the amount of alkaline TGL activity released by perfusion with heparin from the liver of mice with Sarcoma 180 was half that released from the liver of normal mice (11). We found that the hepatic TGL activity is decreased and the LPL activity is increased in a perfusate of tumor-bearing mice.

This paper reports further studies on the decrease in alkaline TGL in the livers of mice with Sarcoma 180.

MATERIALS AND METHODS

Tumor

Sarcoma 180 cells were inoculated intraperitoneally into male ICR/JCL mice (5 weeks old) as described previously (11), and hepatocytes were obtained 10 days later.

Culture of Hepatocytes

Hepatocytes were isolated from normal and tumor-bearing mice using the hepatic portal vein perfusion technique as described by Seglen (12) and Nakamura et al. (13) with slight modifications. The liver was perfused for 5 min at 37 C with calcium- and magnesium-free salt solution (containing 0.137 M NaCl, 5 mM KCl, 0.5 mM $NaH_2PO_4 \cdot 2H_2O$, 0.4 mM $Na_2HPO_4 \cdot 12H_2O$, 10 mM HEPES (N-2′-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), 4 mM $NaHCO_3$, 5 mM EGTA [ethyleneglycol-bis(β-aminoethyl ether) N,N′-tetraacetic acid], 6 μg/ml phenol red•Na, 5 mM glucose, pH 7.2). Next, the liver perfused with the above solution was perfused for 1.5-2 min at 37 C with collagenase solution (containing 0.137 M NaCl, 5 mM KCl, 5 mM $CaCl_2$, 0.5 mM $NaH_2PO_4 \cdot 2H_2O$, 0.4 mM $Na_2HPO_4 \cdot 12H_2O$, 10 mM HEPES, 0.4 mM $NaHCO_3$, 0.5 mg/ml collagenase, 0.05 mg/ml trypsin inhibitor, 6 μg/ml phenol red•Na, pH 7.5). Primary culture of hepatocytes was carried out by the method of Kato et al. (14) with slight modifications. Suspensions of 2-2.5 × 10^6 cells were plated in 60 mm plastic dishes in 4 ml of Eagle's minimum essential medium (MEM) containing 10% fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 25 μg/ml of fungizone and 10^{-8} M insulin, and cultured as monolayers in a humidified atmosphere of 5% CO_2 in air at 37 C. The medium was changed after the first 24 hr and then once every two days. Cell viability was determined by the trypan blue exclusion test.

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Cell Harvesting and Preparation of Cell Homogenate

Cultured hepatocytes were washed twice with 10 mM Tris, 1 mM EDTA (trisodium ethylenediaminetetraacetate, trihydrate), 0.25 M sucrose (pH 7.5). Then the cells were collected in the same buffer using a rubber policeman and centrifuged at 1,000 × g for 5 min. The packed cells were suspended in the same buffer and disrupted by sonication at 0 C in an ultrasonic disruptor (model UR-200P, Tomy Seiko Co., Ltd., Japan) at position 4 for two 5-sec periods.

Incorporation of (^{3}H)leucine into Intra- and Extra-cellular Materials

The medium consisted of Eagle's MEM supplemented with 0.5 μCi/ml of (^{3}H)leucine (specific activity 136 Ci/mmol). Cells in monolayers were harvested with a rubber policeman at the indicated time and washed twice with 10 mM Tris, 1 mM EDTA, 0.25 M sucrose (pH 7.4). TCA (trichloracetic acid)-insoluble materials were prepared from the cells and the medium, and their radioactivities were determined by the method of Tanaka and Ichihara (15).

Measurement of Triglyceride Lipase Activity

TGL activity was measured in the absence of heat-inactivated (56 C, 10 min) human serum with glycerol tri(1-^{14}C)oleate as described previously (11).

DNA Determination

Cellular DNA was measured by the method of Ceriotti (16).

Statistical Analysis

Results are expressed as means ± S.E., and the significance of differences was analyzed by Student's t-test.

RESULTS

Characterization of the Cell Culture System

Freshly isolated hepatocytes from both normal and tumor-bearing mice showed more than 90% viability by the trypan blue exclusion test and became firmly attached to the dishes within 4 to 6 hr after plating. Cells from tumor-bearing mice displayed morphologic characteristics similar to those from normal mice (Fig. 1). Cells cultured in Eagle's MEM yielded homogeneous hepatocyte populations that were free of non-parenchymal cells and Sarcoma 180. Their cellular DNA was relatively stable for a minimum of three days (Fig. 2). The efficiencies of attachment of the cells from normal and tumor-bearing mice after culture for 24 hr were 69.5 ± 2.2 and 65.1 ± 2.6%, respectively, as judged by measurement of DNA.

Both cultured hepatocytes from normal mice and cultured hepatocytes from tumor-

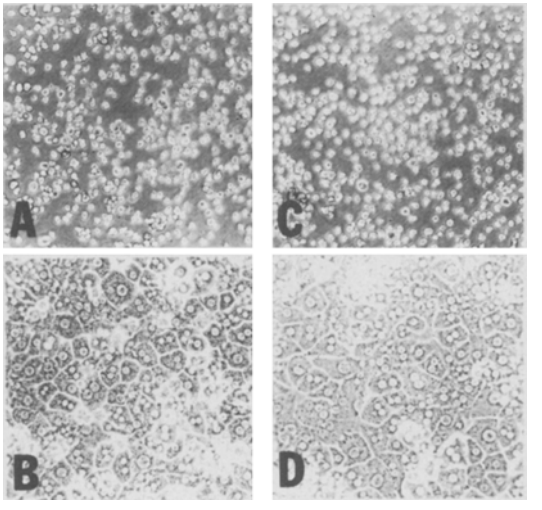

FIG. 1. Phase contrast microscopy of freshly isolated and cultured cells. A and B were cultures of hepatocytes from normal mice on day 0 and 1, respectively. C and D were cultures of hepatocytes from tumor-bearing mice on day 0 and 1, respectively. ×200.

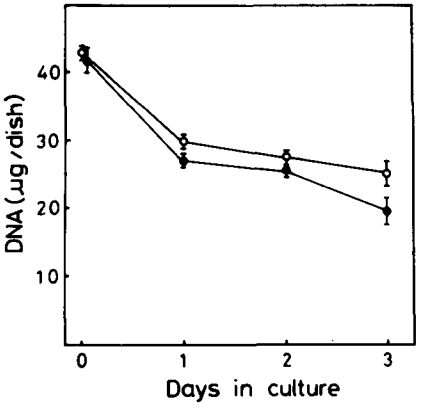

FIG. 2. Cellular DNA in nonproliferating monolayer cultures of hepatocytes. The DNA contents of aliquots of the same cultures of hepatocytes from normal (○) and tumor-bearing (●) mice harvested at different times were determined. Cell culture and assay procedures were as described in Materials and Methods. Bars indicate standard errors of means for 4 experiments.

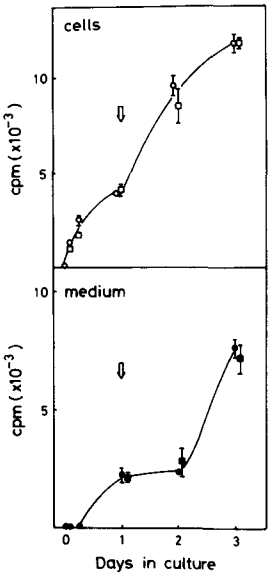

FIG. 3. Incorporation of (^{3}H)leucine into TCA-insoluble materials. Hepatocytes were cultured in the medium consisting of Eagle's MEM supplemented with (^{3}H)leucine (0.5 μCi/ml). The arrow indicates the time of change of medium supplemented with (^{3}H)leucine. Bars indicate standard errors of means for 3 experiments. ○,●, normal mice; □,■, tumor-bearing mice. Open and closed symbols indicate the radioactivities in the cells and medium, respectively.

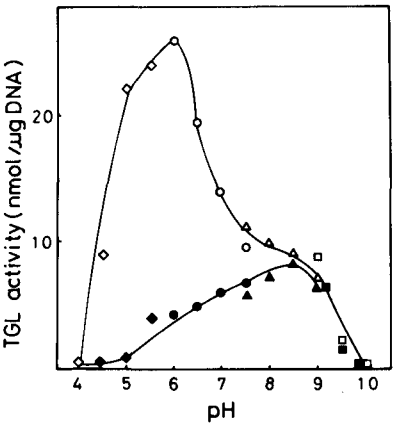

FIG. 4. pH dependence of TGL activity of hepatocytes from normal mice. Incubation was carried out at 37 C for 60 min at the indicated pH in the presence (closed symbols) and absence (open symbols) of 500 μM chlorpromazine without heat-inactivated human serum. Details were as described previously (11). ◇,◆, pH 4.0-5.5, 0.2 M acetate buffer; ○,●, pH 6.0-7.5, 0.2 M potassium phosphate buffer; △,▲, pH 7.5-9.0, 0.2 M Tris-HCl buffer; □,■, pH 9.0-10.0, 0.2 M Na_2CO_3-$NaHCO_3$ buffer.

bearing mice incorporated labeled leucine into TCA-insoluble materials (Fig. 3). TCA label also accumulated in the medium of both cultures. These results suggest that both groups of hepatocytes synthesized and secreted new proteins. The syntheses and secretions of TCA-insoluble materials by hepatocytes from the two sources increased similarly.

TGL Activity of Cell Homogenates

The pH-dependence of the TGL activity of the homogenate of 1-day cultured hepatocytes from normal mice was examined in the absence and presence of chlorpromazine (Fig. 4), which is known to inhibit lysosomal lipases but not alkaline lipases (17). In the absence of chlorpromazine, the lysosomal lipase activity showed a peak between pH 4 and 7, but no clear peak of alkaline TGL activity. On addition of chlorpromazine, the TGL activity between pH 4 and 7 was strongly inhibited, but alkaline TGL activity was only slightly inhibited, and a discrete alkaline pH optimum of TGL activity was observed. Similar profiles of TGL activity as a

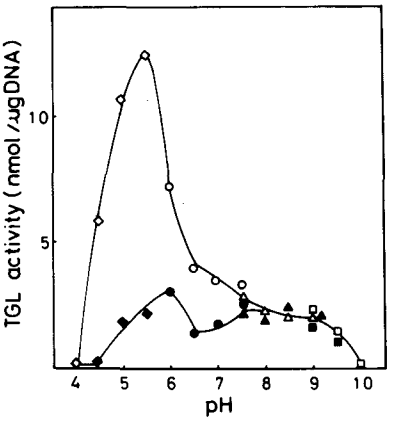

FIG. 5. pH dependence of TGL activity of hepatocytes from tumor-bearing mice. Assay conditions were as described for Fig. 4.

function of pH were obtained with a homogenate of 1-day cultured hepatocytes from tumor-bearing mice (Fig. 5), but the alkaline TGL activity was much less than that in hepatocytes of normal mice. Lysosomal lipase activity also was low in hepatocytes from tumor-bearing mice.

Figure 6 shows the alkaline TGL activity of homogenates of hepatocytes from normal and tumor-bearing mice after 0 to 3 days of culture. Since the partial chlorpromazine inhibition of the TGL activity between pH 7.0 and 9.5 suggests the presence of residual lysosomal lipase activity in this pH range, true alkaline TGL activity should be measured in the presence of chlorpromazine. The TGL activities of the homogenates of cultured hepatocytes from normal and tumor-bearing mice were measured at pH 8.4 in the presence of 500 μM chlorpromazine. The TGL activity of the homogenate of freshly isolated hepatocytes from tumor-bearing mice was 31.7% of that from normal mice (control mice, 7.89 ± 0.45 nmol/μg DNA; tumor-bearing mice, 2.50 ± 0.18 nmol/μg DNA; $p < 0.01$). In cultures from both normal and tumor-bearing mice, the TGL activity increased throughout culture. The TGL activity of a 1-day culture of hepatocytes from tumor-bearing mice was 24.2% of that from normal mice (control mice, 14.43 ± 0.92 nmol/μg DNA; tumor-bearing mice, 3.49 ± 0.29 nmol/μg DNA; $p < 0.01$). The ratio of the TGL activity of the 1-day culture to that of freshly isolated hepatocytes from tumor-bearing mice (1.40 ± 0.06) also was significantly ($p < 0.05$) lower than that of cells from normal mice (1.84 ± 0.12).

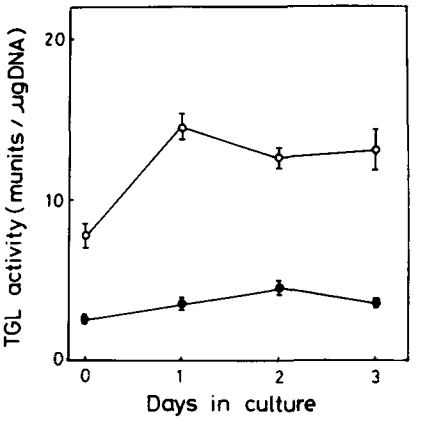

FIG. 6. TGL activity of hepatocytes harvested at different times. Hepatocytes from normal (○) and tumor-bearing (●) mice were harvested at the indicated times. The TGL activity of cell homogenate was measured in the absence of heat-inactivated human serum at pH 8.4 (0.2 M Tris-HCl buffer). Bars indicate standard errors of means for 4 experiments.

DISCUSSION

Previously, we found that in the livers of mice with Sarcoma 180, the hepatic TGL activity decreased with development of the tumor (11). Damen et al. (18) reported that the hepatic TGL activity in post-heparin plasma of mice bearing the GRSL ascites tumor decreased during tumor growth. However, the mechanism by which hepatic TGL decreases in tumor-bearing animals is still unknown.

In the present study, we demonstrated that the alkaline TGL activity of freshly isolated hepatocytes from mice with Sarcoma 180 was one-third that of hepatocytes from normal mice, and that the ratio of the TGL activity of the 1-day cultured hepatocytes to that of freshly isolated hepatocytes from tumor-bearing mice was lower than that of hepatocytes from normal mice (Fig. 6). These results suggest that in the liver of mice with Sarcoma 180, the synthesis of alkaline TGL is impaired. However, this decrease does not seem to be due to a decrease in overall protein synthesis because the incorporation of (^{3}H)leucine into TCA-insoluble materials in hepatocytes of tumor-bearing mice was similar to that in hepatocytes from normal mice (Fig. 3). Labeled TCA-insoluble materials were secreted into the medium, but we could not detect alkaline TGL activity in the medium under the present conditions. Moreover, alkaline TGL activity was not released into the medium by heparin (50 units/ml). However, Nakai et al. (19) reported that triglyceride hydrolase activity was released by heparin from isolated hepatocytes prepared from streptozotocin-diabetic rats.

It is uncertain whether the alkaline TGL in the hepatocytes is hepatic TGL. The alkaline TGL was relatively resistant to NaCl and was inhibited by heat-inactivated human serum (data not shown), suggesting that it has properties similar to hepatic TGL. But the possibility that the alkaline TGL in hepatocytes included LPL activity cannot be excluded. Previously we prepared a liver homogenate of mice three days after inoculation of Sarcoma 180, fractionated it on a heparin-Sepharose column and found that hepatic TGL activity was decreased and LPL-like activity was increased (11). However, when the liver homogenate of mice on day 3 was used as an enzyme source, the TGL activity was inhibited by heat-inactivated human serum (Masuno, H., and Okuda, H., unpublished data). The contribution of LPL-like activity to the alkaline TGL activity of hepatocytes observed in the present study requires further study.

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Mass Spectra of TMS Esters of Deuterated Decanoic Acids and of TMS Ethers of Deuterated Decanols

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ABSTRACT

Mass spectra of trimethylsilyl esters of nine specifically deuterated decanoic acids and of trimethylsilyl ethers of the corresponding deuterated decanols and of tri[$^2H_3$]methylsilyl derivatives of most of the acids and of decanol have been measured. The fragmentation patterns have been used to examine the formation of ions m/z 117, 129, 131, 132, 145, 159, 171, 185 and 201 in the spectrum of the TMS ester of decanoic acid. Mechanisms of breakdown of one ion to another have been proposed. Similarities and differences in ion formation between spectra of TMS esters and methyl esters have been examined. It has been shown that ion m/z 132 is produced by the McLafferty rearrangement and that ion m/z 117 is formed from it by loss of a silyl methyl group. Ion m/z 145 is formed by hydrogen transfer from C-5, C-6 or C-7 to the carbonyl oxygen and γ-cleavage. Ion m/z 129 is formed from ion m/z 145 by loss of the previously transferred hydrogen and a silyl methyl group. A mechanism of formation of ions m/z 115, 129, 143 and 157, which contained only one silyl methyl group, observed in the mass spectrum of the TMS ether of decanol, has been proposed. Ions in spectra of the derivatives can be used to locate the position of deuterium, or other substituents, at a number of the carbons of decanoic acid or decanol.

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INTRODUCTION

Trimethylsilyl esters of alkanoic acids are readily prepared and may be employed in GC-MS analyses instead of methyl esters (1). Effective use of TMS esters in determining structure requires elucidation of the mode of formation of the major ions in the spectra. However, there have been few investigations of these ions. The principal ions in spectra of TMS esters have been described (1) and some of them, which also appear as minor ions in spectra of bis TMS esters of dicarboxylic acids, have been discussed (2). Mass spectra of TMS esters of deuterated 4-phenoxybutyric acids also have been examined (3), but there do not seem to have been any other investigations.

Origins of major ions in mass spectra of methyl esters have been determined from spectra of methyl esters of deuterated octadecanoic acids (4-6). An investigation of the mass spectra of TMS esters of all the gem dideuterated decanoic acids and of [10-$^2H_3$]decanoic acid, which were prepared for other purposes, now have been carried out. The fragmentation patterns of the ions have been analyzed by comparing the spectra of the various deuterated analogs and also of the tri[$^2H_3$]methylsilyl esters. Study of the origin of the mass spectroscopic fragments is useful in examining the products of new syntheses of deuterated acids where location of deuterium may be uncertain and in determining structure of long-chain acids in general.

Mass spectra of the TMS ethers of specifically deuterated decanols, corresponding to the deuterated acids mentioned above, also have been studied. It was hoped that the spectra would include ions which also would show deuterium position and thus complement the results provided by spectra of TMS esters of acids. The spectrum of the TMS ether of n-decanol first was reported nearly 30 yrs ago (7), but there has been only one investigation of the ions using deuterated TMS ethers. Spectra of TMS ethers of five specifically deuterated pentanols were described, but the larger ions which are of interest in spectra of longer chain TMS ethers were absent (8).

EXPERIMENTAL PROCEDURES

The nine specifically deuterated acids were prepared as reported before (9,10). [10-$^2H_3$], [9-$^2H_2$], [8-$^2H_2$] and [7-$^2H_2$] decanols were synthesized as described previously (9); [2-$^2H_2$], [3-$^2H_2$], [4-$^2H_2$], [5-$^2H_2$] and [6-$^2H_2$] decanols were prepared by lithium aluminum hydride reduction of the corresponding acids (10), and [1-$^2H_2$]decanol was obtained by lithium aluminum deuteride reduction of methyl decanoate. Isotopic purities of the alcohols were the same as those of the acids reported previously (10); [1-$^2H_2$]decanol was 99.5% dideuterated. TMS esters of acids and ethers of alcohols were prepared by treating a solution of acid or alcohol in methylene chloride with bis (trimethylsilyl)acetamide just prior to GC-MS analysis. Tri[$^2H_3$]methylsilyl ethers were pre-

pared in the same way using tri[$^2H_3$]methylchlorosilane (MSD Isotopes, Dorval, Quebec, Canada). The tri[$^2H_3$]methylsilyl esters of the acids were obtained by allowing the reagent to react with acids in dry pyridine solution for 24 hr at 25 C. Electron impact GC-MS data were measured with a model 4000 Finnigan GC-MS system interfaced with a model 2300 Finnigan Incos data acquisition system. The source temperature was 250 C, and the ionization potential was 70 eV. The GC column was a 60 m × 0.32 mm fused silica column coated with DB-5 and connected directly to the ion source. The linear velocity of the helium carrier gas was 40 cm/s. Samples were injected in the splitless mode at an initial temperature of 50 C, and the temperature was raised ballistically to 125 C and programmed at 4 C/min to 250 C.

RESULTS AND DISCUSSION

The mass spectrum of the TMS ester of decanoic acid is shown in Figure 1; the intensities of the ions were as follows: 229 [32], 201 [2], 185 [2], 171 [0.5], 159 [2], 145 [10], 132 [18], 131 [14], 129 [19], 117 [62], 75 [92], 73 [100]. The $[M-15]^+$ ion (m/z 229), formed by loss of a silyl methyl group, is well known in spectra of TMS esters of acids (1-3), and formation of ions m/z 73 $[(CH_3)_3\overset{+}{S}i]$ and 75 $[(CH_3)_2Si{=}\overset{+}{O}H]$ has been studied previously (3). The ions which may be useful in structure determination are those with m/z 117, 129, 131, 132, 145 and the weak ions 159, 171, 185 and 201. Mass spectra of methyl alkanoates contain two major ions m/z 74 and 87 (4,11,12), and it has been proposed that the ions m/z 132 and 145, mentioned above, are the TMS analogs of these methyl ester ions because of the mass difference of 58 amu $[OSi(CH_3)_3$ compared to $OCH_3]$ (1,2). For this reason, also, ion m/z 201 was thought to be analogous to ion m/z 143 in the methyl ester spectrum (2). Ion m/z 131 and the minor ions mentioned above have not been discussed before.

Spectra of the TMS esters of all the specifically deuterated decanoic acids were recorded and the principal ions compared with those of the unlabelled ester in Table 1. MS of the tri[$^2H_3$]methylsilyl esters of decanoic acid and of [2-$^2H_2$], [3-$^2H_2$], [4-$^2H_2$], [5-$^2H_2$] and [6-$^2H_2$] decanoic acids also were measured, with the results listed in Table 2. Comparison of these ion-labelling results with those observed in spectra of methyl esters of deuterated acids (5,10) established that ion m/z 132 is in fact the TMS analog of ion m/z 74 which is the base peak in spectra of methyl alkanoates (11). Ion

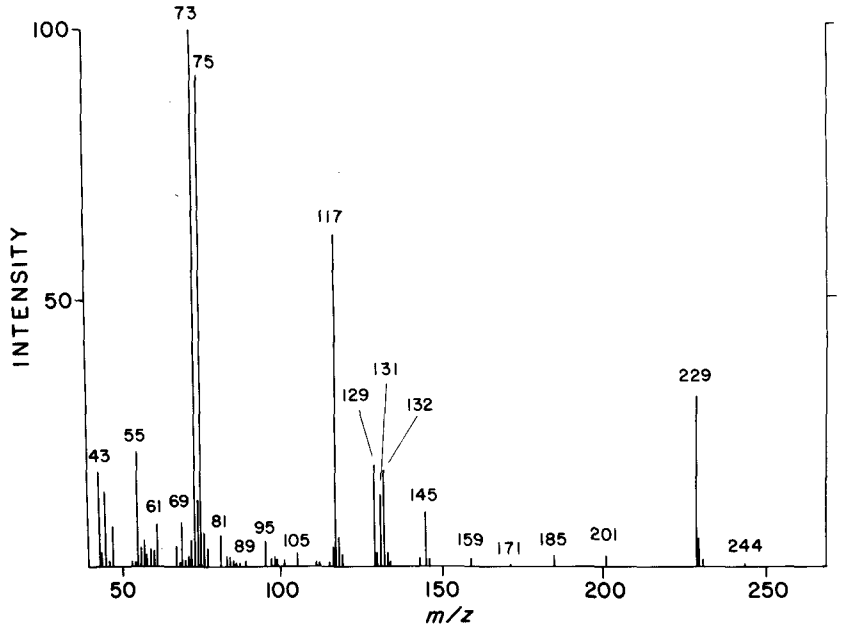

FIG. 1. Mass spectrum of the TMS ester of decanoic acid.

TABLE 1

Principal Ions (m/z) in Mass Spectra of TMS Esters of Undeuterated and Specifically Deuterated Decanoic Acids*

Undeuterated ester	Deuterium position								
	2-$^2H_2$	3-$^2H_2$	4-$^2H_2$	5-$^2H_2$	6-$^2H_2$	7-$^2H_2$	8-$^2H_2$	9-$^2H_2$	10-$^2H_3$
75	76	75	75	75	75	75	75	75	75
117	119	117	117 118	117	117	117	117	117	117
129	130 (129)	131	129	129	129	129	129	129	129
131	133	133	131	132	131	131	131	131	131
132	134	132	133	132	132	132	132	132	132
145	145 (146)	147	145	145 146	145 146	145 (146)	145	145	145
159	161 (160) (159)	159 (160) (161)	161	159	159	159	159	159	159
171	173	173	173	173 (171)	173	171 (172) (173)	171 (172) (173)	171 (173)	171 (173)
185	187	187	187	187	187	187	187	187	188
201	203 (202) (201)	203 (201)	203 (201)	203	203 (201) (202)	203 (202)	203 (201)	201 (203)	201 (204)
229	231	231	231	231	231	231	231	231	232

*When two or more deuterated ions are listed, those in parentheses are less intense.

TABLE 2

Comparison of m/z of Ions in Mass Spectra of TMS Esters and Tri[$^2H_3$]methylsilyl Esters of Deuterated and Undeuterated Decanoic Acids*

TMS ester	Tri[$^2H_3$]methylsilyl esters					
		Deuterium position				
Decanoic acid	Decanoic acid	2-$^2H_2$	3-$^2H_2$	4-$^2H_2$	5-$^2H_2$	6-$^2H_2$
75	81	82	81	81	81	81
117	123 124	125 126	123 124	124	123 124	123 124
129	135	136 (135)	137	135	135	135
131	137	139	139	137	138	137
132	141	143	141	142	141	141
145	154	154 (155)	156	154	154 155	154 155
159	168	170	168	170	168	168
171	177	179	179	179	179	179
185	191	193	193	193	193	193
201	210	212 (211)	212 (210)	212 (210)	212	212
229	235	237	237	237	237	237

*Less intense ions are in parentheses.

m/z 132 appeared at m/z 133 in the spectrum of the TMS ester of [4-$^2H_2$]decanoic acid (Table 1), showing that it is formed by the McLafferty rearrangement (12). In this rearrangement, as shown in Figure 2a, a deuterium is transferred from C-4 to the ester carbonyl and subsequent β-cleavage gives ion m/z 133. This mechanism is confirmed by the appearance of this ion at m/z 134 in the spectrum of the TMS ester of [2-$^2H_2$]decanoic acid. Similar mechanisms were proposed previously to explain the mass spectrum of methyl alkanoates (4,11,12).

The labelling pattern in Table 1 also showed that ion m/z 145 is formed as depicted in Figure 2b for the spectrum of the TMS ester of [3-$^2H_2$]decanoic acid, where this ion increased to m/z 147. In spectra of esters of [2-$^2H_2$], [5-$^2H_2$], [6-$^2H_2$] and [7-$^2H_2$] decanoic acids both ions m/z 145 and 146 were observed. In fragmentation of the latter three esters, deuterium is transferred from either C-5, C-6 or C-7 to the carbonyl oxygen, reciprocal hydrogen transfer then produces a radical site on the α-carbon and γ-cleavage gives the above two ions. This is the same mechanism that was used to explain formation of ion m/z 87 in spectra of methyl alkanoates (4-6) and confirms the suggestion that ion m/z 145 is the TMS analog of this ion.

It was assumed that ion m/z 117 was formed from ion m/z 132 by loss of a silyl methyl group (3) so that in the spectrum of the TMS ester of [4-$^2H_2$]decanoic acid ion m/z 133 should yield ion m/z 118. However, both ions m/z 117 and 118 with equal intensities were observed (Table 1). A possible mechanism is shown in Figure 3a, migration of a hydrogen from a TMS methyl group to the carbonyl oxygen is followed immediately by loss of either a CH_3 or a CDH_2 group producing the two ions. The fact that the ions produced are of equal intensity suggests that transfer of hydrogen from the silyl methyl group to the carbonyl oxygen and cleavage of CH_3 or CDH_2 groups occur at approximately the same rate. This mechanism was supported by the spectra of the tri[$^2H_3$]methylsilyl esters (2) of the acids

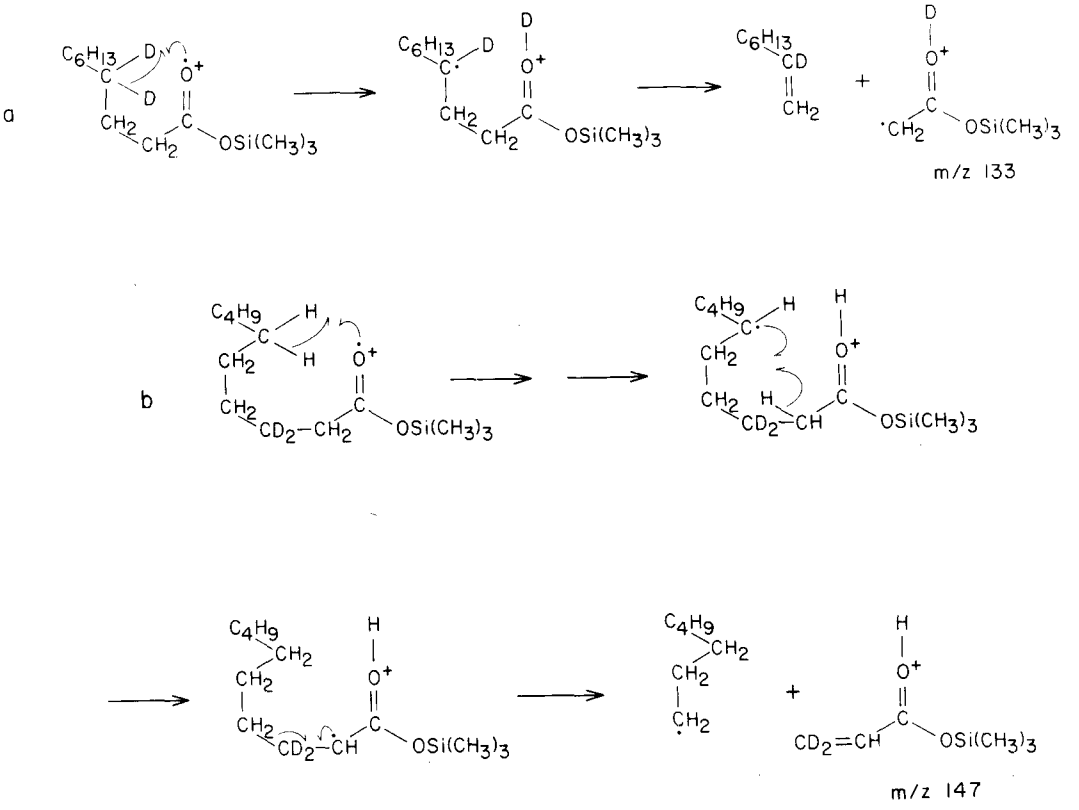

FIG. 2. a. Formation of ion m/z 133 in the mass spectrum of TMS ester of [4-$^2H_2$]decanoic acid. b. Formation of ion m/z 147 in the mass spectrum of TMS ester of [3-$^2H_2$]decanoic acid.

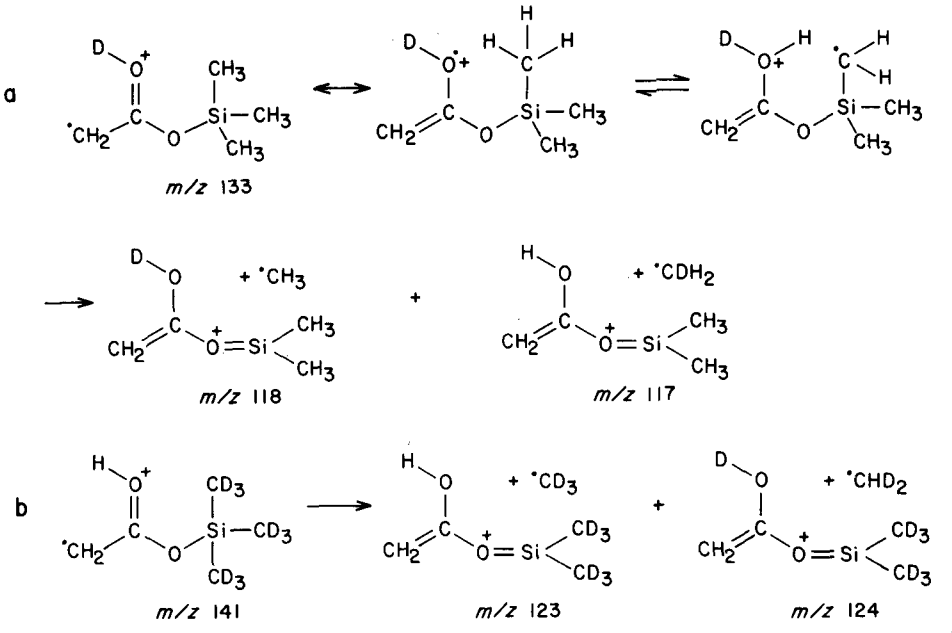

FIG. 3. a. Proposed mechanism of breakdown of ion m/z 133 in the mass spectrum of TMS ester of [4-$^2H_2$] decanoic acid. b. Breakdown of ion m/z 141 in the mass spectrum of tri[$^2H_3$] methylsilyl ester of decanoic acid.

(Table 2); use of this derivative also confirmed that ion m/z 117 contains only two methyl groups attached to silicon. Breakdown of ion m/z 141, in the spectrum of the tri[$^2H_3$] methylsilyl ester of decanoic acid, to ions m/z 123 and 124 with loss of CD_3 or CHD_2 groups, respectively, is shown in Figure 3b. The MS of the tri[$^2H_3$] methylsilyl ester of [4-$^2H_2$] decanoic acid in which only the ion m/z 124 appeared (Table 2) provided further confirmation because here deuterium, transferred from C-4, is attached to carbonyl oxygen and no exchange between hydrogen and deuterium (as in Fig. 3b) is possible.

The results in Tables 1 and 2 also showed that ion m/z 129 is derived from ion m/z 145 by loss of a silyl methyl group and a hydrogen. It increased in mass by 1 and 2 amu, respectively, in spectra of TMS esters of [2-$^2H_2$] and [3-$^2H_2$] decanoic acids but was unchanged in spectra of esters of [5-$^2H_2$], [6-$^2H_2$] and [7-$^2H_2$] acids. Therefore, as illustrated in Figure 4, in the case of the ester of [5-$^2H_2$] decanoic acid, the hydrogen lost is that on the carbonyl oxygen which originally migrated from C-5 (C-6 or C-7 for the other isomers).

It was not possible to propose a mechanism for formation of ion m/z 131. Ions m/z 146 or 147 from which it might have been derived, with loss of CH_3 or CH_4 respectively, in a manner analogous to the mechanisms just discussed, were not observed. This ion became ion m/z 133 in the spectra of esters of [2-$^2H_2$] and [3-$^2H_2$] decanoic acids and ion m/z 132 in spectra of the ester of [5-$^2H_2$] decanoic acid, but was not labelled in the spectrum of any other deuterated ester. These labelling patterns were partly obscured by the McLafferty ion, m/z 132, but spectra of the tri[$^2H_3$] methylsilyl esters confirmed the above results and showed that only two silyl CH_3 groups were present in ion 131. Thus, in spectra of the deuterated derivatives of [2-$^2H_2$] and [3-$^2H_2$] decanoic acids, ion 131 appeared as ion 139 and in the spectrum of the corresponding ester of [5-$^2H_2$] decanoic acid as ion m/z 138. Because of the loss of a CD_3 group in formation of these ions there was no interference by the McLafferty ion, with three CD_3 groups at m/z 141, in spectra of these deuterated derivatives. Deuterium is therefore retained at C-2 and C-3 and a deuterium migrates from C-5; the structure $DCH_2CH_2COO^+{=}Si(CH_3)_2$ is proposed for this ion (m/z 132) as it appears in the spectrum of the TMS ester of [5-$^2H_2$] decanoic acid.

The labelling pattern of ion m/z 159 was very similar to that of ion m/z 101 in the spectrum of methyl decanoate, so that it is most

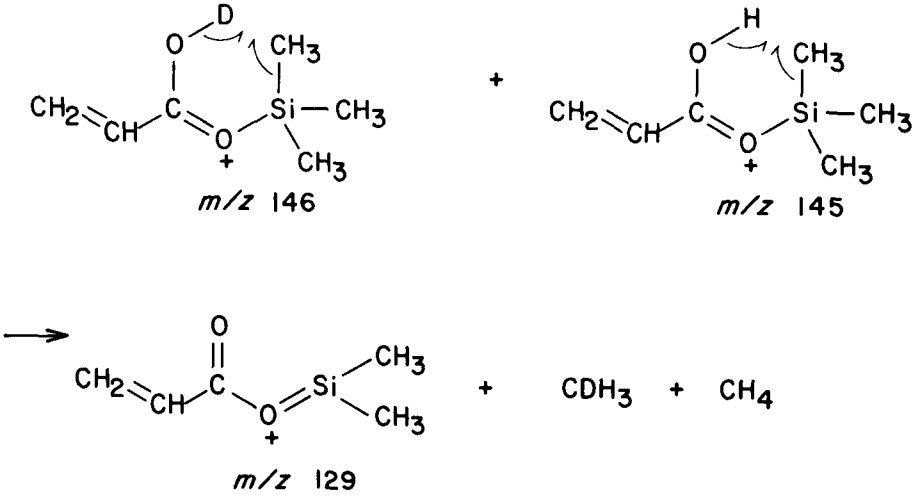

FIG. 4. Breakdown of ions m/z 146 and 145 in the mass spectrum of TMS ester of [5-$^2H_2$] decanoic acid.

probably formed in an analogous manner by cleavage between C-4 and C-5 (10). Ions m/z 173 and 187, which would be similar to ions m/z 115 and 129 in MS of methyl esters (10), were too weak to be investigated. Ion m/z 201 appeared in part as the deuterated ion m/z 203 (or 204 in the case of the [10-$^2H_3$] ester) and in part was unchanged in spectra of esters of all the deuterated isomers except in the spectrum of the TMS ester of [5-$^2H_2$] decanoic acid in which only ion m/z 203 was present (Table 1). This is exactly the same labelling pattern as that observed in the mass spectra of methyl deuterated decanoates (10), showing that the two ions are formed in similar ways.

Ion m/z 201 is thus formed in two ways, by cleavage of the C-7 to C-8 bond (6) leading to ion m/z 203 in spectra of the [2-$^2H_2$] to [7-$^2H_2$] isomers, and by expulsion of C-2, C-3, C-4 and a hydrogen (4,11,12), giving ion m/z 203 in spectra of the [5-$^2H_2$] to [9-$^2H_2$] isomers (and to ion m/z 204 in the spectrum of the [10-$^2H_3$] isomer). Ions m/z 171 and 185 differed from the above ions in that they contained only two of the TMS CH_3 groups (Table 2). The origin of these ions is unclear; the latter cannot be derived from ion 201 by loss of CH_4 because it retains deuterium completely for every deuterated ester, quite different from changes observed in ion m/z 201 (Table 1).

The mass spectrum of the TMS ether of decanol is shown in Figure 5, and relative intensities of the ions were 215 [77], 157 [2], 143 [2], 129 [3], 115 [7], 103 [43], 89 [33], 75 [100] and 73 [71]. The spectra of the tri[$^2H_3$] methylsilyl ether of decanol and of TMS ethers of all the specifically dideuterated decanols and of [10-$^2H_3$] decanol also were measured, and the masses are listed and compared in Table 3. The changes in ions m/z 75 [$(CH_3)_2Si{=}\dot{O}H$], 89 [$(CH_3)_2HSi\dot{O}{=}CH_2$] and 103 [$(CH_3)_2Si\dot{O}{=}CH_2$] were the same as those observed when spectra of TMS ethers of deuterated pentanols were examined (8). Those spectra showed that ion m/z 75 was formed by nonspecific hydrogen transfer from C-1, C-2, C-3 and C-4 and perhaps to a small extent from C-5. The present spectra, however, showed further that there was no contribution to this ion of hydrogens from carbons C-6 to C-10. Ions m/z 89 and 103 increased by two amu in the spectrum of the TMS ether of [1-$^2H_2$] decanol as expected.

The series of minor ions, m/z 115, 129, 143 and 157, are of interest and have not been reported before. The spectrum of the tri[$^2H_3$] methylsilyl derivative showed that these ions retained only one of the silyl methyl groups. A possible mode of formation of these ions from the M-15 ion is shown in Figure 6. A hydrogen is transferred to silicon from C-3, C-4, C-5 or C-6 and the carbon-carbon bond between carbons α- and β- to the radical site is broken. There is some scrambling of hydrogens on the carbons of the bond next to the one which is broken, but the spectrum of the TMS ether of [4-$^2H_2$] decanol, for example where ions m/z 130 and 131 (n=3) are equally intense, showed that much of the deuterium on C-4, one atom of which was transferred to silicon, was retained.

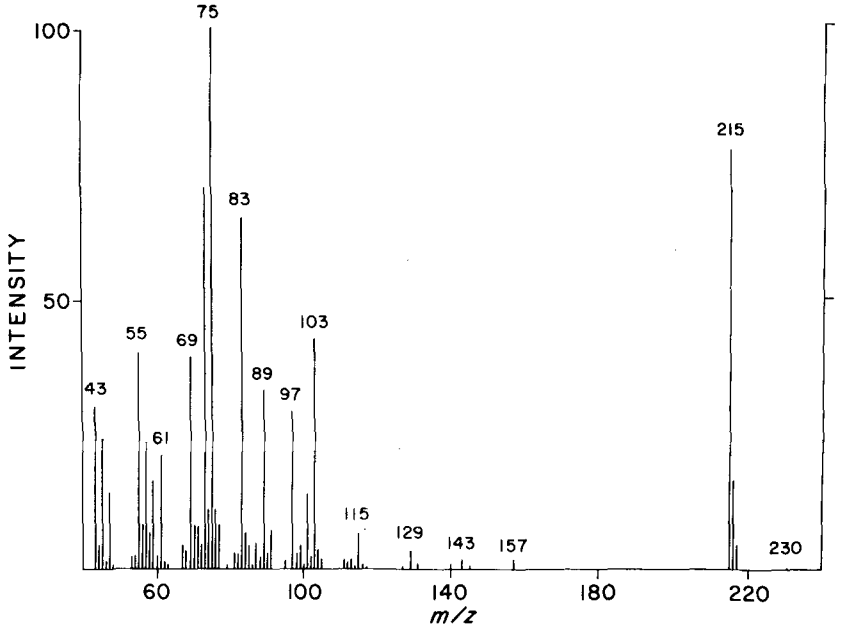

FIG. 5. Mass spectrum of TMS ether of decanol.

TABLE 3

Comparison of m/z of Ions in Mass Spectra of TMS Ethers of Decanol and Deuterated Decanols*

Decanol TMS ether	Decanol tri[$^2H_3$]-methylsilyl ether	Deuterated decanols as TMS ethers									
		Deuterium position									
		1-$^2H_2$	2-$^2H_2$	3-$^2H_2$	4-$^2H_2$	5-$^2H_2$	6-$^2H_2$	7-$^2H_2$	8-$^2H_2$	9-$^2H_2$	10-$^2H_3$
73	82	73	73	73	73	73	73	73	73	73	73
75	81	75 (76)	75 (76)	75 (76)	75 (76)	75	75	75	75	75	75
89	95	91	89	89 (90)	89	89	89	89	89	89	89
103	112	105	103	103	103	103	103	103	103	103	103
115	118	117	117	117 (115)	115 117	115	115	115	115	115	115
129	132	131	131	131	130 131	129 (131)	129	129	129	129	129
143	146	145	145	145	145	145	145 143	143	143	143	143
157	160	159	159	159	159	159	159	159	157	157	157
215	221	217	217	217	217	217	217	217	217	217	218

*Less intense ions are in parentheses.

This examination of the MS of deuterated TMS esters and ethers has established the origin of most of the important ions observed and hence indicates how structural modification would affect the spectra. Since relative intensities of ions are often variable, it is best that

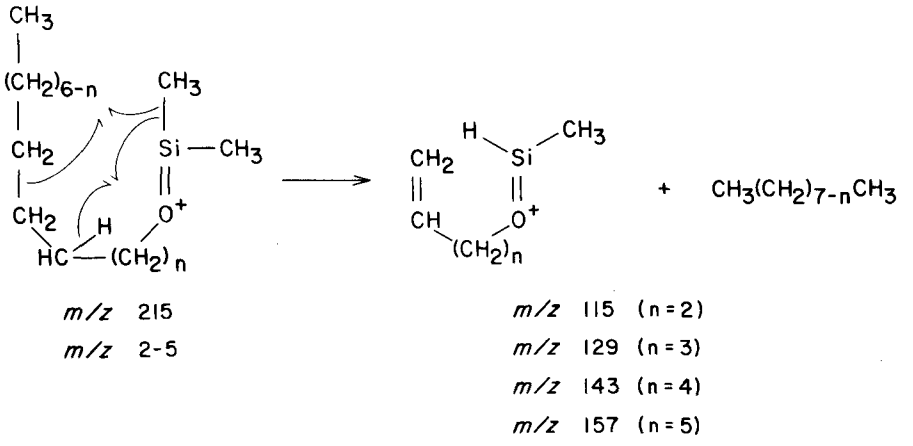

FIG. 6. Proposed formation of ions m/z 115, 129, 143 and 157 from the M-15 ion in the mass spectrum of TMS ether of decanol.

a particular structure, or substituent position such as that of deuterium, is indicated by changes in the mass of several ions. This is in fact the case, as is shown by Tables 1 and 3, where changes at carbons 2, 3 or 4 would affect a number of ions. The spectrum of the TMS ester of (E)-2-docosenoic acid is an example; new strong ions with m/z 155 and 143 were present, and ion m/z 117 was relatively weak (13).

Structural changes involving carbons 5, 6 or 7 would cause less specific changes in spectra of TMS esters, except for the effect of deuterium at C-5 on ion m/z 131 (Table 1). Ions m/z 115, 129, 143 and 157 in spectra of TMS ethers of alcohols are, however, affected by changes at these positions as shown in Table 3. Changes in the masses of these ions can indicate the position of deuterium up to carbon 7. Since ion m/z 201 in spectra of TMS esters is formed both by cleavage of the C-7 to C-8 bond and by expulsion of C-2 to C-4 and a hydrogen (4,11, 12), modification of the last three carbons affects this ion, changes at C-10 having the most pronounced effect (Table 1).

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Uptake of Secondary Autoxidation Products of Linoleic Acid by the Rat

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ABSTRACT

Incorporation of secondary autoxidation products (SP) of linoleic acid into the rat body was investigated. Radioactive SP was administered orally to a group of 5 rats, and excretions of radioactive substances in feces, urine and respiration were measured and compared with excretions from rats fed linoleic acid and its hydroperoxides. The SP-fed group excreted 45% and the other groups about 10% of the administered radioactivity through feces. Urinary excretion accounted for 52% of activity ingested in the SP group and less than 30% in the other groups. The $^{14}CO_2$ produced in each group was about 25% of the ingested activity. Incorporation of the radioactive substances of SP into tissues and organs was measured periodically after administration of a single dose. The radioactive substances accumulated in the liver between 12-24 hr after administration and accounted for 2.6% of the total amount given, the highest level of all tissues and organs. This accumulation led to an elevation of serum transaminase activities, an increase in hepatic lipid peroxide, as determined by thiobarbituric acid test, and a slight hypertrophy of liver (1.5-fold). Therefore, absorbed SP appeared to contribute to the deleterious condition of the liver.
Lipids 20:412-419, 1985.

INTRODUCTION

Lipid peroxides are formed endogenously by active oxygen species (1-3). The significant biological and deleterious roles of lipid peroxides in vivo have been investigated in medicine and biology (4-11). Polyunsaturated fatty acid such as linoleic acid (LA) reacts easily with atmospheric oxygen to form linoleic acid hydroperoxides (LAHPO). The LAHPO is broken down in the presence of oxygen (12) and subsequently forms a mixture of further oxidized products (13). The mixture generally is termed secondary autoxidation products (SP). Many data indicate that both LAHPO and SP are toxic. The toxicity of SP is due to various kinds of compounds such as polymers (14), aldehydes (15) including malonaldehyde (MA) (16), hydroperoxy aikenals (17), and hydroperoxy epoxides (18). These substances occur in our daily food, though their amounts may not always be high. The incorporation of these substances and their effects on animals are important problems. Studies have been done concerning the gastrointestinal absorption of LA (19) and incorporation of LAHPO into the animal body (20-24). Although SP is the predominant component in the autoxidation products of LA (13) and may be more toxic than LAHPO (15,25), the extent of incorporation of SP into the animal body is not clear. In the present study, the incorporation of SP administered intragastrically was compared radiochemically with that of LA and LAHPO using materials uniformly labeled with ^{14}C. In particular, the effect of SP on liver was examined.

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MATERIALS AND METHODS

Radioactive Autoxidation Products of LA

[U-^{14}C]LA (14.0 MBq), purchased from New England Nuclear, USA, was diluted with nonradioactive LA (Tokyo Kasei Kogyo Co., Ltd.) to a specific activity of 172 kBq/mmol. The LA was autoxidized at 37 C for 7 days under air. LA, LAHPO and SP fractions were obtained from the autoxidized LA by silica gel column and thin layer chromatography (TLC) (13). These chemicals were identified by their weights, radioactivity, functional group concentrations: carboxy (26), peroxy (27,28) and aldehyde groups (29), and thiobarbituric acid (TBA) test (see below).

Animals

Male Wistar albino rats, 5 weeks old and each weighing about 110 g (Clea Japan, Inc.), were housed at approximately 23 C with a light and dark cycle of 12 hr. The diet was prepared daily and its peroxide value was maintained at less than 0.5 meq/kg. It consisted of 30% sucrose, 24% corn starch, 25% casein, 15% soybean oil, 4% McCollum's salts mixture, 1% cellulose powder and 1% vitamin mixture (2500 IU retinyl palmitate, 2.5 mg thiamine hydrochloride, 3.5 mg riboflavin phosphate, 2.5 mg pyridoxine hydrochloride, 25 mg nicotinamide, 10 mg panthenol, 75 mg ascorbic acid, 250 IU

calciferol and 100 mg lysine hydrochloride in the 1 ml and was purchased from Shionogi and Co., Ltd.). After feeding for 1 week, the animals weighing 157-162 g were selected and separated into groups. Food was withheld for about 4 hr and each rat was given a single dose of the LA, LAHPO or SP intragastrically at about 6:30 pm using tuberculin syringes connected to the gastric tube. The amounts administered were determined by measuring the radioactivity remaining in the syringes. The radioactivity administered to each rat was converted to a standard of 46.4 kBq. The rats were then placed individually in sealed glass boxes (metabolism cages) and given diet and water ad libitum. Air from the box was slowly removed by a vacuum pump into a bottle containing 25 ml of monoethanolamine.

Radioassay of Animal Excreta

The monoethanolamine bottle trapping $^{14}CO_2$ was changed at regular intervals. In triplicate, 3 ml of the monoethanolamine was transferred into a vial and dissolved with 9 ml methanol in 8 ml of a scintillation cocktail composed of 6 g PPO and 0.5 g POPOP in 1 liter of toluene. Urine was washed periodically into a beaker and absorbed by cellulose sheets. Feces also were collected on cellulose sheets. These sheets were weighed and combusted by a sample oxidizer (Packard Model 305 Tri-Carb). Radioactivity was measured with a scintillation counter (Rack Beta 1215, LKB Wallac), and the counting efficiency of each vial was determined by an external standard. Recovery of radioactivity with the sample oxidizer was 97.2 ± 1.2% (n=10). A higher efficiency was obtained by this procedure than by the direct use of a scintillator containing solubilizer.

Measurement of Radioactivity Incorporated into Animal Tissues and Organs

The radioactive SP (46.4 kBq each) was administered orally to 40 rats. Five rats were picked at random every regular interval after administration, stunned by a cephalic blow and bled from the carotid artery. Tissues and organs were excised, perfused or washed with saline solution, weighed, and cut into small pieces. A sample of these pieces, in triplicate, was wrapped with cellulose sheets and combusted by the sample oxidizer. Some of the hepatic pieces were examined by the TBA test, while blood was assayed for transaminase activities by the Karmen method (30).

TBA Test

TBA reactive substances (TBARS) were assayed by the method of Asakawa and Matsushita (31) with a few modifications. The hepatic pieces were homogenized in saline solution with a teflon homogenizer. A 2-ml aliquot of the homogenate was added to the TBA-reaction mixture consisting of 2 ml TBA reagent (0.5% TBA in 50% acetic acid), 2 ml acetic acid and 0.1 ml butylated hydroxytoluene (25 mM in ethanol). The mixture was saturated with nitrogen and heated for a short period (15 min) to avoid a concomitant autoxidation of hepatic lipids. After centrifugation, optical density of the supernatant was measured at 532 nm and compared with the results of nontreated rats. The difference in optical density was presented as a μeq value of MA after conversion, with a calibration curve of authentic MA. The amounts of TBARS in feces (wet weight) and in urine (color intensity of creatinine by Jaffe reaction) also were measured.

Statistical Analysis

The Student's t test was used to determine statistical significance. The variability of the data is presented as mean ± SE.

RESULTS

Effects of Autoxidation Products of LA on Rat Growth

Purities of LA and the prepared LAHPO were 98 and 93%, respectively (Table 1). The SP fraction was composed roughly of 35% polymers, 25% endoperoxide-rich components and 40% lower molecular-weight components. The concentration of the aldehyde group in SP was about 2/5ths that of the carboxy group, and the major aldehydes were azelaldehydic acid (4.8%) and hexanal (3.7%). When the chemicals were administered orally to rats at several dosage levels (Fig. 1), 3 consecutive administrations of 700 mg/day of SP induced a remarkable depression in body weight and resulted in the death of the rats on the fourth day. Rats receiving a dosage of more than 200 mg of LAHPO or 700 mg of SP exhibited significant growth impairment ($P<0.001$), reduction in food consumption and diarrhea on the first day. These animals recovered from the stress the following day. No difference in body weight gain was detected among groups receiving no dose, the single dose of 300 mg of LA, 100 mg of LAHPO, and 200 mg of SP, when a 0.05 probability level was chosen. All of these animals were clinically normal. Five rats given intraperitoneal injections of 100 mg of SP also survived. Therefore, in the following experiments, 100 mg each of these chemicals was used for oral doses.

TABLE 1

Chemicals for Administration

Analysis	Chemicals		
	LA	LAHPO	SP
μeq of: carboxy group	352	303	292
peroxy group	ND	283	67.8
aldehyde group	–	–	127
as MA	ND	4.01	0.968
Radioactivity (kBq)	60.5	53.8	46.4
% of radioactivity in: polymers			36
endoperoxides			26
lower molecular-weight components			38
azeladehydic acid			4.8
hexanal			3.7
azelaic acid			2.5
monocarboxylic acids			2.0
suberaldehydic acid			0.8

Radioactive LA, LAHPO and SP (100 mg each) were identified as mentioned in the text. Furthermore, SP was separated by Sephadex LH-20 gel filtration into 3 parts: polymer-rich, endoperoxide-rich and lower molecular-weight components. Their content percentages were calculated by the radioactivities. The second part was UV inactive and peroxide reaction positive (32). Dihydroxystearate derivatives were detected from hydrogenated silylated materials of the second part by mass spectrometry. Therefore, the second part was considered to be endoperoxide-rich. The third part was subjected to gas chromatography and the major components were quantified as described previously (12). Monocarboxylic acids were mainly hexanoic, heptanoic and octanoic acids.

Comparison of Excretion of Radioactive Substances by SP-, LA- and LAHPO-fed Groups

Figure 2 shows fecal excretion of radioactive substances from SP-, LA- and LAHPO-fed groups. These three groups exhibited similar excretion patterns (peaks at 20 hr after administration), but the SP group had a secondary minor peak at 7 hr. The amount of TBARS per radioactivity in the minor peak was 0.154 ± 0.015 μeq/7240 Bq, while the amount in SP per se was 0.968 μeq/46400 Bq (Table 1), i.e., 0.151 μeq/7240 Bq. Although diarrhea was never observed in the SP group, this ratio indicated that the minor peak was excreted SP per se. Figure 3 shows that urinary excretion patterns of radioactive substances of these 3 groups were similar to each other. The peroxide value in the urine of the LAHPO group was negative. A small amount of TBARS was detected in the urine of the SP group. Figure 4 shows radiorespirometric patterns of the 3 groups. The SP group had an expiration rate with 2 peaks around 5 and 15 hr, whereas the LA and LAHPO groups indicated single peaks at 10 and 7 hr, respectively. Then, SP was separated by Sephadex LH-20 into molecular-weight components higher (SP-H) and lower (SP-L) than the molecular weight of LAHPO, and it also was reduced by sodium borohydride (SP-R). When the SP-H, SP-L and SP-R were administered orally to 2 rats each, the radiorespirometric pattern of the SP-L rats tended to differ from that of the SP-H rats (Fig. 5). SP-H rats gave a single peak at 4 hr, and SP-L rats gave 2 peaks, at 4 hr and 12 hr. SP-R rats excreted $^{14}CO_2$ faster than the above rats.

Total Percentage of Radioactivity Incorporated into Body

The total radioactivity excreted by each rat until 90 hr after administration was calculated (Table 2), because a low level of radioactivity could be detected in excreta of all rats even after 75 hr. The amount of radioactive substances excreted through feces by the SP group was larger than that from any other group. When administered radioactivity minus excreted activity to feces was defined as the amount of radioactive substances retained in the body, these incorporated substances were 95% in the LA, 85% in the LAHPO and 55% in the SP groups. Discharge of radioactive substances by urination increased significantly in the order of LA < LAHPO < SP groups with the SP group excreting about half of the activity incorporated into body. The production of $^{14}CO_2$ was pro-

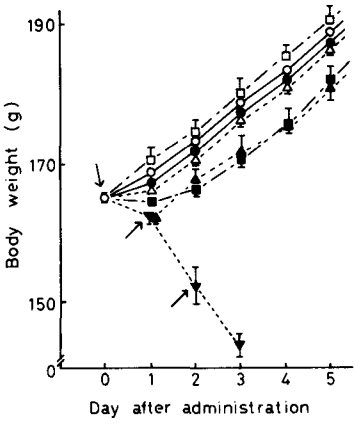

FIG. 1. Effects on rat growth from oral administration of LA, LAHPO and SP. The rats were separated into 8 groups of 10 rats each. To determine the administered amounts, a part of radioactive LA, LAHPO or SP (333 Bq) was added to the cold materials before administration. These chemicals were given orally at points indicated by arrows. One group was bled without any chemicals as a reference group (—○—). Another group was administered 110-140 mg of LA and grew similarly to the reference group. The other groups received the following doses of chemicals: 250-350 mg of LA (—●—), 75-120 mg of LAHPO (-·-□-·-), 190-230 mg of LAHPO (-·-■-·-), 180-230 mg of SP (- - -△- - -), 650-750 mg of SP (- - -▲- - -), and about 700 mg/day of SP for 3 consecutive days (- - -▼- - -). The last group exhibited a mortality of 100% on the fourth day.

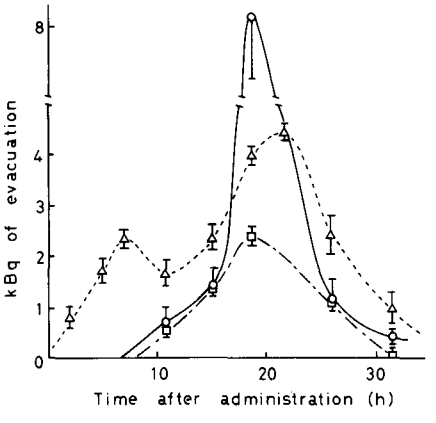

FIG. 2. Changes in radioactivity in feces of rats orally administered labeled LA, LAHPO and SP. The rats were divided into 3 groups of 5 rats each and given doses of LA (—○—), LAHPO (-·-□-·-) and SP (- - -△- - -). The feces of each rat was collected at 4.3, 5.5, 8.5, 13, 17, 20, 23, 29 and 34.5 hr after administration. Mean values ± SE of the radioactivity of feces were calculated and plotted at each midpoint of the sampling times.

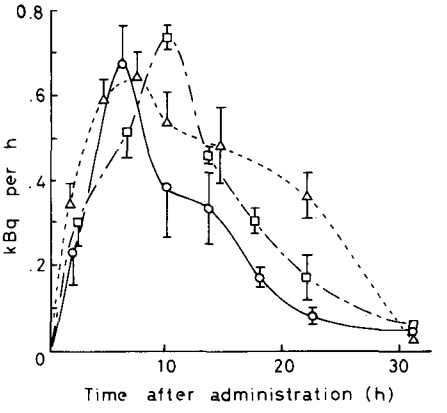

FIG. 3. Changes in radioactivity in urine of rats orally administered labeled LA (—○—), LAHPO (-·-□-·-) and SP (- - -△- - -). The radioactivity in urine of each rat was assayed at about 4-hr intervals until 24 hr and at 38 hr after the administration. The activity was divided by the interval times (hr) and plotted at each midpoint of the sampling times.

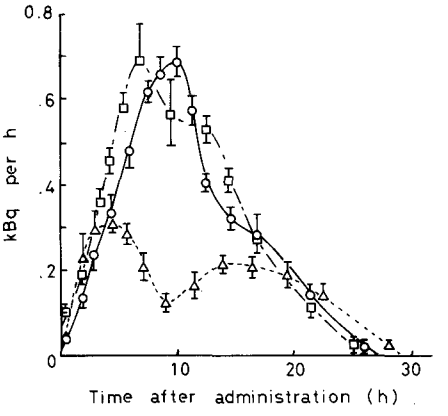

FIG. 4. Radiorespirometric patterns of rats orally administered labeled LA (—○—), LAHPO (-·-□-·-) and SP (- - -△- - -). The respiratory radioactivity was measured at regular intervals, divided by the interval times (hr) and plotted at each midpoint of the sampling times.

portionately the same (25%) for every group. No remarkable differences were observed among the excretion percentages of SP-H, SP-L and SP-R rats.

Incorporation of Radioactive Substances into Liver in SP Group

Rats given single doses of SP were exsanguinated at regular intervals and radioactivity in the tissues and organs was measured (Table 3).

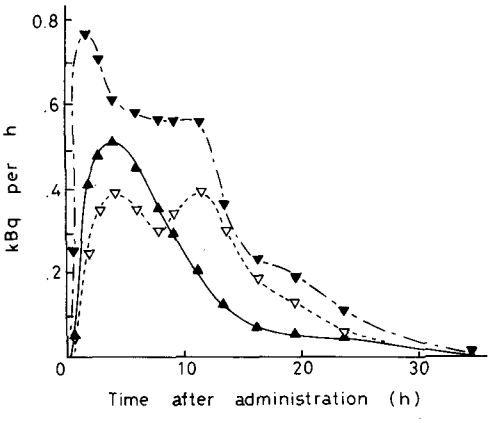

FIG. 5. Radiorespirometric patterns of SP-H (—▲—), SP-L (- - -△- - -) and SP-R (—·—▼—·—). The time course was observed following the same procedure as in the case of SP.

The radioactive contents in intestinal lumen, which probably were SP, increased with a decrease in radioactive contents in gastric lumen. The activity of the intestinal contents reached a maximum at 6 hr after administration and decreased to 1% of the given total at 72 hr. The change in radioactivity of blood exhibited 2 peaks at 3 hr and 24 hr. Incorporation of the radioactivity into the intestine also exhibited 2 peaks, a major one at 6 hr and a secondary one at 24 hr. Changes in the incorporated radioactivity in brain, lung, spleen, kidney and testis were parallel to that in intestine. The radioactivity in fat pads, pancreas and femoral muscle gave a peak at 24 hr. In liver, the radioactivity clearly increased and reached a maximum at around 12 hr. The quantity in liver was 2.6% of the dose and the greatest of all tissues and organs. Next, a change in hepatic lipid peroxide content was measured with the TBA test (Fig. 6). The lipid peroxide content rose with the increase in radioactivity until 24 hr. After the rapid decline of radioactivity, the lipid peroxide content still remained at the high level, which was accompanied by increases in serum GOT and GPT activities and a slight hypertrophy (about 1.5-fold; $P<0.001$).

DISCUSSION

Deteriorated substances in fats are a complex mixture of autoxidation products. In the pres-

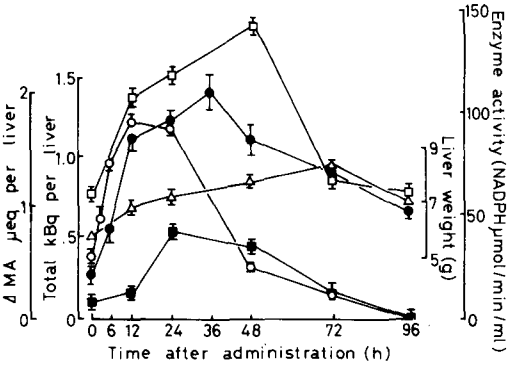

FIG. 6. Effect of oral intake of SP on liver. The radioactivity incorporated into liver (—○—) is given in Table 3. Liver weight (—△—), lipid peroxide content (—●—) and serum transaminase activities–glutamic oxaloacetic transaminase (GOT; —□—) and glutamic pyruvic transaminase (GPT; —■—)–were measured concurrently with assay of radioactivity incorporation.

TABLE 2

Total Radioactivity Recovered from Excreta of Rats Orally Administered Labeled Chemicals

	Administration of:					
	LA	LAHPO	SP	SP-H	SP-L	SP-R
	Mean ± SE with 5 rats			Mean of 2 rats		
Weight of feces (mg)	2120 ± 980	2250 ± 300	5860 ± 220	1730	2030	3810
Recovered % of administered amount (46.4 kBq) from feces	6.4 ± 1.7	13.4 ± 1.9	45.3 ± 1.0	23	18	25
Recovered % of the respective absorbed amount into body						
from urine	17.2 ± 2.7	29.3 ± 1.8	51.9 ± 0.8	22	38	24
from respiration	22.4 ± 1.9	23.9 ± 1.2	22.9 ± 0.7	16	17	31

Radioactivity from feces, urine and respiration excreted by each rat (Fig. 2) until 90 hr after administration was totaled.

TABLE 3

Incorporation Amounts of ^{14}C from Orally Administered SP into Rat Organs and Tissues

	Mean ± SE (n=5) of total radioactivity (Bq) per whole organ or tissue						
	Hr after feeding						
	0.5	3	6	12	24	48	72
Brain	51 ± 9	44 ± 2	79 ± 14	55 ± 9	72 ± 7	56 ± 6	35 ± 4
Lung	49 ± 6	60 ± 5	88 ± 7	53 ± 1	114 ± 13	86 ± 10	48 ± 11
Heart	17 ± 1	15 ± 1	30 ± 3	20 ± 1	20 ± 1	20 ± 2	19 ± 1
Blood (per ml)	10 ± 2	166 ± 18	31 ± 3	57 ± 6	184 ± 25	32 ± 1	47 ± 3
Stomach	1080 ± 130	455 ± 84	341 ± 145	120 ± 47	150 ± 18	66 ± 12	14 ± 1
Gastric content	35470 ± 6493	23249 ± 3406	13273 ± 155	12050 ± 1629	881 ± 198	126 ± 23	50 ± 15
Intestine	295 ± 47	508 ± 85	903 ± 153	437 ± 80	624 ± 65	192 ± 19	67 ± 7
Intestinal content	894 ± 311	4242 ± 551	7375 ± 663	4473 ± 531	4412 ± 508	2136 ± 264	560 ± 55
Pancreas	26 ± 5	25 ± 2	23 ± 4	22 ± 2	35 ± 6	24 ± 2	13 ± 1
Liver	397 ± 43	632 ± 65	960 ± 25	1212 ± 61	1179 ± 102	323 ± 26	168 ± 14
Spleen	16 ± 2	12 ± 1	47 ± 18	21 ± 1	26 ± 3	22 ± 2	10 ± 1
Kidney	77 ± 5	73 ± 2	143 ± 24	78 ± 12	92 ± 7	60 ± 3	54 ± 2
Perirenal fat pad	63 ± 13	60 ± 7	65 ± 14	90 ± 9	103 ± 24	81 ± 4	68 ± 4
Epididymal fat pad	49 ± 16	52 ± 11	60 ± 6	89 ± 9	109 ± 22	63 ± 3	47 ± 4
Testis	44 ± 5	31 ± 4	84 ± 13	50 ± 7	56 ± 4	41 ± 5	29 ± 4
Femoral muscle	32 ± 2	30 ± 6	32 ± 3	28 ± 6	35 ± 9	24 ± 2	19 ± 2

In 96 hr rats, only liver was radioassayed. The results are shown in Fig. 6.

ent study, SP which is a mixture of aldehyde-rich products (Table 1) was obtained from autoxidized LA and administered orally to rats, as were LA and LAHPO.

The single oral dose of 200 mg of LAHPO resulted in no more than diarrhea and growth impairment to the rats on the first day (Fig. 1). Holman and Greenberg (33) demonstrated that the intraperitoneal LD_{50} of ethyl linoleate hydroperoxide for mice is 12 mg and that the oral dose does not kill. Bergan and Draper (20) reported that oral [1-^{14}C] methyl linoleate hydroperoxide is not absorbed per se into the animal body. Nakatsugawa and Kaneda (21) showed that only 0.23% of methyl linoleate hydroperoxide in the orally administered total is detected in rabbit lymph by high performance liquid chromatography. Thus, it is believed that orally fed LAHPO is readily reduced to nontoxic substances such as hydroxy fatty acids in the animal gastrointestinal tract. On the other hand, although a single dose of 700 mg of SP produced only stress in the animals on the first day, 3 daily injections resulted in the death of the animals on the fourth day. The single dose of a smaller amount (100 mg) had no effect on the animals, whether administered orally or through intraperitoneal injections. It is suggested that SP is not as toxic as LAHPO but may be absorbed per se.

The SP-administered group excreted about half of the given activity through feces (Table 2). The total fecal weight was also 3 times the weight of the LA group. SP seemed to have an exfoliation effect on brush border membrane such as that exhibited by some kinds of detergents (34). The remaining half was incorporated into the body, and 1/2 was discharged by urination and 1/4 was exhalted. Thus, radioactive substances in the SP group were metabolized to CO_2, as also seen in the LA and LAHPO groups.

While the rates of excretion through both feces and urine in the SP group were almost the same as those of the LA and LAHPO groups (Figs. 2 and 3), the radiorespirometric pattern of the SP group was different from those of the other groups (Fig. 4). The radiorespirometric pattern of the SP group gave 2 peaks and was similar to that of the SP-L rats (Fig. 5). SP-L might comprise 2 components. One was rapidly metabolized to CO_2, while the other was not.

SP-H was composed mainly of polymers of LA. No remarkable differences were observed in either respiration or excretion between SP-H and SP-L rats (Table 2). The molecular-weight range of SP-H was determined to be about 1000-300 by Sephadex LH-20 gel permeation. It is believed that SP-H could be absorbed by the rats, although thermally oxidized oil which was the higher molecular-weight polymer was not (35).

The peak of accumulated radioactivity in tissues and organs was in agreement with the second peak of the radiorespirometric pattern (Table 3). The content of hepatic lipid peroxide remained at a high level after the radioactivity disappeared. Since elevation of serum transaminase activities and a hypertrophy of liver also were detected (Fig. 6), it is suggested that SP, whose form was at least partly unchanged, was incorporated into liver, metabolized, contributed to hepatic impairment and then was transferred to the other tissues and organs.

The present study demonstrates that about 1/2 of the orally fed SP is incorporated into the rat body, where 1/2 is excreted through urine and 1/4 through CO_2. The remaining part of SP is metabolized in the liver, where it leads to a deleterious condition. The possible toxicity of SP may be due to aldehyde compounds (36,37).

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Effect of KCD-232, a New Hypolipidemic Agent, on Serum Lipoprotein Changes in Hepatoma-Bearing Rats

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ABSTRACT

Changes in serum lipoprotein profiles were characterized in Donryu rats subcutaneously implanted with an ascites hepatoma line of AH109A cells and the effect of a new hypolipidemic agent with a structure of 4-(4'-chlorobenzyloxy)benzyl nicotinate (KCD-232) was estimated. With the growth of hepatoma for periods of up to three weeks, a striking decrease in the high density lipoprotein (HDL) fraction and an enormous increase in the VLDL+LDL (very low density lipoprotein + low density lipoprotein) fraction were found in hepatoma-bearing rats when either precipitation method or electrophoresis was used. These lipoprotein profiles were not influenced by sex. Judging from the electrophoretogram, the increase in VLDL+LDL fraction was due mainly to an increase in LDL fraction. The oral administration of KCD-232 significantly suppressed the hepatoma-induced increase in VLDL+LDL with little or no influence on the hepatoma-induced decrease in HDL. There existed a positive correlation between the hepatoma weight and (VLDL + LDL)-cholesterol concentration and a negative one between hepatoma and HDL-cholesterol.

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INTRODUCTION

It has been reported that several hepatomas show a loss of normal inhibition of Ch (cholesterol) biosynthesis by Ch feeding (1-3) and a defect in the dietary regulation of FA (fatty acid) biosynthesis (4,5). The hepatoma often induced hypercholesterolemia in rats (6,7) and humans (8,9) as do other tumors, such as a pituitary tumor in rats (10). In the rat, serum lipoprotein patterns are altered depending on the malignant properties of Morris hepatomas (11). Another study by Schneider et al. reported that estrone, an inhibitor of Ch biosynthesis, retarded the growth of Novikoff hepatoma subcutaneously inoculated into rats (12). KCD-232, a new compound with a structure of 4-(4'-chlorobenzyloxy)benzyl nicotinate, has hypocholesterolemic (13) and hypotriglyceridemic (14) activities in rats. Its hypocholesterolemic activity is based on the inhibition of both Ch absorption from the intestine and Ch synthesis in the liver (15). The hypotriglyceridemic property of the drug depends on the inhibition of hepatic triglyceride synthesis due to both decreased FA synthesis and increased FA oxidation in the liver (16). It has been well known that certain hypolipidemic drugs such as clofibrate, nafenopin and others induce peroxisome proliferation in the liver and increase peroxisomal β-oxidation (17-19). In contrast to these compounds, some hypolipidemic drugs such as vitamin B_2-butyrate, nicomol, ML-236B, KF1492 and pantethine have been reported to exert little influence on the hepatic peroxisomal enzymes. It has been suggested that the participation of hepatic peroxisomes in hypolipidemic activities of these drugs may be little if any (20). The action mechanism of KCD-232 seems to be different from those of peroxisome proliferators, because KCD-232 neither increases the activities of cyanide-insensitive FA oxidation (16) nor catalase (15), one of the marker enzymes of peroxisomes, nor induces the proliferation of hepatic peroxisomes in electron microscopic studies (15,16). The presence of the nicotinic acid moiety in the KCD-232 molecule may partly explain the inability of KCD-232 to proliferate hepatic peroxisomes, since the nicotinate derivative, nicomol, shows no influence on peroxisomal enzymes such as catalase, urate oxidase, fatty acyl-CoA oxidizing system or carnitine acetyltransferase (20). Moody et al. recently have reported an inverse biological association between liver catalase activity and serum Ch concentration in tumor-bearing rats treated with peroxisome-proliferating hypolipidemic agents (21). Although KCD-232 seems not to be a peroxisome proliferator, it has an inhibitory action, like estrone, on Ch biosynthesis. In the present study, therefore, we have focused our attention on the effect of KCD-232 on tumor growth and serum Ch metabolism, especially on hepatoma-induced abnormalities in serum lipoprotein profiles, using Donryu rats bearing an ascites hepatoma line of AH109A.

MATERIALS AND METHODS

Animals and Tumor

Female Donryu rats 8 to 10 weeks of age were used throughout these experiments; where indicated, male rats of the same strain and age

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were used. Animals were housed in an air conditioned room and fed a stock pellet (CE-2, CLEA Japan Inc., Tokyo) ad libitum. Rat ascites hepatoma line of AH109A was provided by the Sasaki Institute (Tokyo). This tumor was maintained in Donryu rats by the weekly intraperitoneal transfer of ascitic fluid (0.5-1.0 ml). Ascitic fluid was harvested from the animals as needed and tumor cells were collected by low speed centrifugation at 1000 rpm and 4 C for 10 min, washed three times with phosphate-buffered saline, pH 7.4, and resuspended in the same buffer. Viable cells were counted with a Bürker-Türk hemocytometer using trypan blue exclusion and diluted with the same buffer to obtain a density of 5×10^7 cells/ml. To produce a solid form of AH109A, subcutaneous implantation of 5×10^6 tumor cells per rat was used. A sham implantation was performed in tumor-free rats. To observe the effect of KCD-232, the drug suspended in an aqueous solution of 0.5% carboxymethyl cellulose was given orally to tumor-bearing rats at a daily dose of 30 mg/0.5 ml/100 g body weight. In this experiment, normal (tumor-free) and control (tumor-bearing) rats were given orally only 0.5% carboxymethyl cellulose solution.

Sample Preparation

Rats were deprived of their diet at 9 a.m. and killed by decapitation at 1 p.m. on scheduled days. In the experiment to observe drug efficacy, the final administration of KCD-232 also was performed at 9 a.m. Blood was collected in a glass tube and left to clot at room temperature. The solid tumor was removed quickly, dissected free from the surrounding connective tissues and washed with cold 0.9% NaCl solution. It was blotted on filter paper and weighed. The serum was obtained by centrifugation and kept at 4 C for Ch and lipoprotein analyses.

Lipoprotein Separation and Cholesterol Determination

Serum lipoproteins were separated into VLDL+LDL and HDL fractions by a slight modification of the precipitation method described by Burstein et al. (22) and Lopes-Virella et al. (23). To 0.2 ml of serum, 2 ml of the solution containing 0.02% sodium phosphotungstate and 50 mM $MgCl_2$ were added. After standing for 20 min, centrifugation was performed for 15 min at 800 x g. The precipitate was dissolved in 2 M NaCl, diluted with distilled water and used for the determination of Ch content of VLDL+LDL fraction. The supernatant was used for Ch determination of the HDL fraction. Completeness and selectivity of precipitation by this procedure were assessed by electrophoresis. Total Ch contents of unfractionated whole serum (S-Ch), VLDL+LDL fraction [(VLDL+LDL)-Ch] and HDL fraction (HDL-Ch) were determined by an enzymatic method using a Determiner TC "555" kit (Kyowa Hakko Kogyo Co., Ltd., Tokyo).

Lipoprotein Electrophoresis

Lipoprotein electrophoresis was carried out on cellulose acetate plates (TITAN III-LIPO, Helena Laboratories, Beaumont, Texas) following the directions of the supplier. Each run was performed at 200 V constant voltage for 20 min and stained with oil red 0.

Statistical Methods

The statistical analysis was performed using the Student's t-test for paired data, and a P value of <0.05 was considered significant. The correlation coefficient (r) was determined by linear regression analysis using a computer.

RESULTS

Changes in the serum Ch levels were first examined in female rats who had borne the hepatoma for 3 weeks (Fig. 1). The serum total Ch (S-Ch) level of these animals was not elevated but was almost the same as that of tumor-free (normal) rats. Estimation of Ch distribution among lipoproteins, however, revealed a marked increase in (VLDL+LDL)-Ch (360% of normal) and decrease in HDL-Ch (43% of normal) in the tumor-bearing rats; this resulted in a notable increase in (VLDL + LDL)-Ch/HDL-Ch ratio (960% of normal). Though not shown, almost the same results were obtained in male Donryu rats bearing the hepatoma, thus indicating that these abnormalities of serum lipoprotein profile were not influenced by sex in this animal.

To examine how the lipoprotein profiles changed as time proceeded, normal (N), tumor-bearing (T) and tumor-bearing, KCD-232-treated (TK) female rats were sacrificed every week for three weeks. As illustrated in Figure 2, the (VLDL+LDL)-Ch level of T rats increased linearly during this period with significant differences (at least $P < 0.05$) from N rats at all time points. On the other hand, the HDL-Ch level of T rats was unchanged for the first week, and then declined approximately linearly. KCD-232 had no effect on the decrease in HDL-Ch level, whereas it significantly inhibited the elevation of the (VLDL+LDL)-Ch level at all time points. Though the S-Ch level of T rats rose only slightly as time went on, a slight rise in the third week was significantly different from the level of N rats. KCD-232 significantly decreased the S-Ch level in T rats in the second and third weeks by reducing the (VLDL+LDL)-Ch

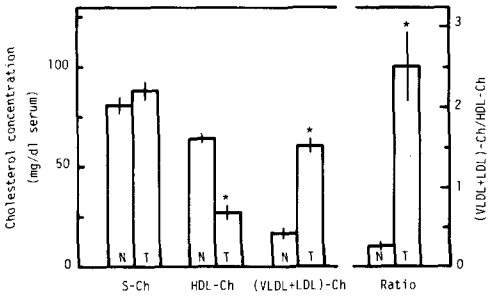

FIG. 1. Changes in serum lipoprotein profiles in normal (N) and tumor-bearing (T) rats. Female Donryu rats were killed 3 weeks after the subcutaneous implantation of AH109A (5×10^6 cells/rat). Serum lipoproteins were separated into VLDL+LDL and HDL fractions by the precipitation method, and concentrations of whole serum cholesterol (S-Ch), HDL-Ch and (VLDL+LDL)-Ch were determined by enzymatic method. Each value represents the mean of 6 rats. Vertical bars indicate standard errors. *P < 0.001 to the N group.

level. Though not shown, the (VLDL+LDL)-Ch/HDL-Ch ratios of N rats were almost constant for three weeks, whereas those of T rats were significantly higher at all time points. The ratios of TK rats were always lower than those of T rats.

Figure 3 shows an electrophoretogram of serum lipoproteins from N, T and TK rats. The serum samples from two different animals of each group were applied to the plate. The dark HDL and light LDL bands observed in N rats were reversed in T rats, that is, these animals had the light HDL and dark LDL bands. The dark band of LDL in T rats became faint with KCD-232 administration (TK rats), while the drug slightly deepened the light band of HDL in T rats. Judging from the electrophoretogram, the increase in the VLDL+LDL fraction of T rats (Fig. 2) was considered to be due mainly to an increase in the LDL fraction.

It seemed that the (VLDL+LDL)-Ch concentration increased and the HDL-Ch decreased

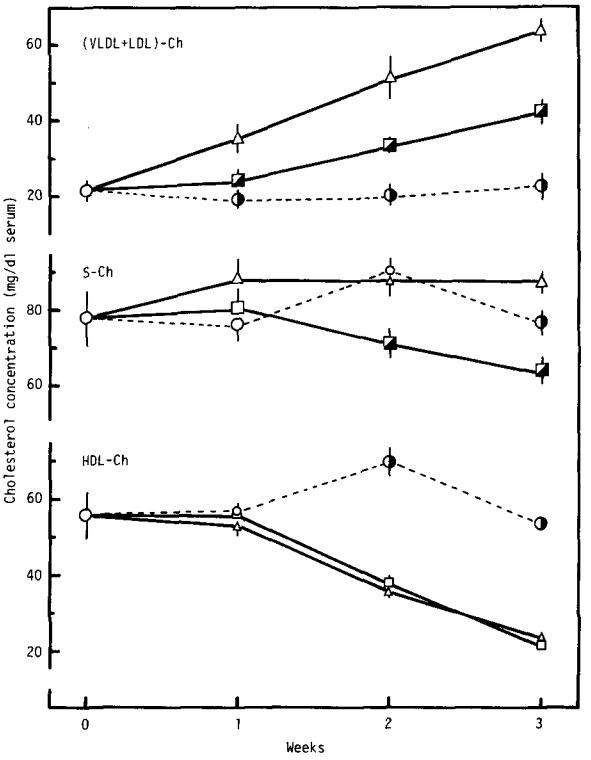

FIG. 2. Changes with time in serum lipoproteins in normal (N), tumor-bearing (T) and tumor-bearing, KCD-232-treated (TK) rats. Female Donryu rats (200-230 g) were subcutaneously implanted with AH109A (5×10^6 cells/rat) and killed every week for 3 weeks. See legend to Fig. 1 for determination of S-Ch, HDL-Ch and (VLDL+LDL)-Ch. Each point represents the mean of 5-10 rats. Vertical bars indicate standard errors. (○), N group; (△), T group; (□), TK group (300 mg/kg/day of KCD-232). Half closed circle (◑) and square (◪) indicate significant differences from T group (△), at least P < 0.05.

with growth of the tumor. This led us to carry out linear regression analyses of tumor weight vs. fractional Ch concentrations. The results are shown in Table 1. A positive correlation was noticed in a comparison of tumor weight and (VLDL+LDL)-Ch and a negative one between tumor weight and HDL-Ch. No significant correlation was observed between tumor weight and S-Ch, because the two lipoprotein fractions counterbalanced each other. KCD-232 significantly ($P < 0.05$) suppressed the initial growth of hepatoma; the solid hepatoma weights of T and TK rats in the first week were 1.4 ± 0.3 (6) and 0.7 ± 0.2 (6) g/rat (mean ± SEM for the number of rats indicated in parentheses), respectively. Thereafter the suppressive effect of the drug gradually diminished; the hepatoma weights of T and TK rats in the second week were 15.0 ± 1.7 (5) vs. 10.7 ± 1.6 (5), and those in the third week were 34.2 ± 3.5 (10) vs. 33.9 ± 3.9 (7).

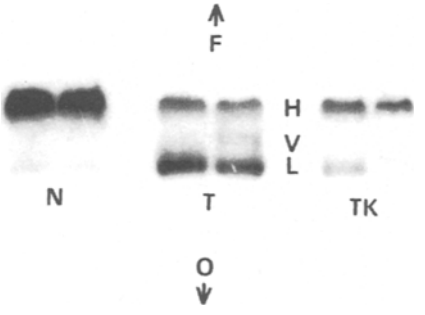

FIG. 3. Cellulose acetate plate electrophoretogram of serum lipoproteins from normal (N), tumor-bearing (T) and tumor-bearing, KCD-232-treated (TK) rats the third week following implantation of AH109A. The serum samples (0.9 μl) from 2 different female Donryu rats of each group were applied to the plate, which was run at 200V constant voltage for 20 min and stained with oil red 0. Abbreviations: V, very low density lipoproteins; L, low density lipoproteins; H, high density lipoproteins; O, origin; F, front.

DISCUSSION

In the present study, serum lipoprotein profiles were characterized in Donryu rats bearing a hepatoma line of AH109A, and the effect of KCD-232 on them was examined. With tumor growth, lipoprotein profiles in the serum from AH109A-bearing rats indicated a striking decrease in HDL-Ch and an enormous increase in (VLDL+LDL)-Ch. Judging from an electrophoretogram, the increase in (VLDL+LDL)-Ch seemed due mainly to the increase in LDL fraction. This, however, remains to be confirmed by ultracentrifugation analysis. Though the S-Ch level showed scarcely any rise, about 10-fold elevation was found in the ratio of (VLDL+LDL)-Ch to HDL-Ch, which might be an index for considering atherogenicity. These changes were not influenced by sex. The oral administration of KCD-232 significantly sup-

TABLE 1

Variable Linear Regression Analyses of Hepatoma Weight Versus Fractional Cholesterol Concentration

Variable	n	r	P	Equation
Hepatoma vs (VLDL+LDL)-Ch				
T group	21	0.735	<0.001	Y = 0.722X + 37.8[a]
TK group	18	0.738	<0.001	Y = 0.477X + 25.6
Both groups	39	0.655	<0.001	Y = 0.673X + 31.2
Hepatoma vs HDL-Ch				
T group	21	-0.886	<0.001	Y = -0.764X + 50.8
TK group	18	-0.829	<0.001	Y = -0.854X + 51.9
Both groups	39	-0.855	<0.001	Y = -0.803X + 51.3
Hepatoma vs S-Ch				
T group	21	0.083	<0.500	Y = 0.025X + 86.3
TK group	18	-0.464	<0.100	Y = -0.376X + 77.5
Both groups	39	-0.112	<0.500	Y = -0.097X + 81.4

[a]X and Y represent hepatoma weight (g/rat) and fractional cholesterol concentrations (mg/dl serum), respectively, of tumor-bearing (T) and tumor-bearing, KCD-232-treated (TK) female Donryu rats.

pressed the tumor-induced increase in VLDL+LDL with little or no influence on the tumor-induced decrease in HDL, resulting in a fall of the value in (VLDL+LDL)-Ch/HDL-Ch. In normal rats, KCD-232 also reduced the (VLDL+LDL)-Ch strongly and HDL-Ch weakly (15).

High density lipoproteins, including HDL_2, have been reported to decrease in humans with cancer (24,25), in rats with Walker carcinosarcoma 256 or tumors induced by 9,10-dimethyl-1,2-benzanthracene (26) and in mice bearing Ehrlich ascites tumors (27). The results of the present study with AH109A are in good agreement with these findings. However, Narayan previously reported elevations in HDL, LDL and VLDL concentrations of rats bearing tumors which were induced by feeding N-2-fluorenylacetamide, a hepatocarcinogen (7). Dnistrian et al. also have reported an increase in HDL_2 concentration in rats with Morris hepatoma 16 and 7777 (11). The present results obviously are not in agreement with their findings, despite the use of liver tumors in these studies. There is no clear explanation for this discrepancy at present, but differences in the degree of differentiation of hepatomas and/or the different biochemistries which might be associated with each hepatoma may be one explanation, as HDL_2 was not elevated in the serum of rats bearing Morris hepatoma 5123TC (11).

There existed a positive correlation between the tumor weight and (VLDL+LDL)-Ch concentration and a negative one between the tumor weight and HDL-Ch concentration (Table 1). These results are somewhat helpful in understanding the cause(s) of changes in lipoprotein profiles of AH109A-bearing rats, for it is possible to interpret the two correlations as follows: The host serum (VLDL+LDL)-Ch concentration may rise depending on the tumor growth, while HDL-Ch may diminish gradually from the host serum with growth of the tumor. It was suggested that liver tumors were capable of synthesizing serum lipoproteins, but that nonhepatic tumors apparently did not have this ability (7). Hence the increase in VLDL+LDL fraction may be explained by overproduction of LDL via VLDL, or LDL as such by AH109A. KCD-232 may be assumed to inhibit the syntheses of VLDL and/or LDL in the hepatoma and/or host liver, as Ch synthesis from [^{14}C]acetate in slices of both solid hepatoma and host liver was inhibited by oral administration of KCD-232 to AH109A-bearing rats (unpublished data). The particularly intriguing question of why HDL decreases in the host serum remains to be answered. As proposed by Creinin and Narayan, who reported the reduction of HDL in mice bearing Ehrlich ascites tumors (27), HDL might be utilized in the formation of cellular membranes of AH109A cells. Further studies must be performed to verify this highly speculative hypothesis and to clarify the exact mechanism(s) for the action of KCD-232.

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Effect of Brominated Vegetable Oils on Heart Lipid Metabolism[1]

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ABSTRACT

Normal rats fed for 105 days on an experimental diet made up of standard laboratory chow supplemented with 0.5% of a mixture of brominated sunflower-olive oil (BVO) developed a significant increase in the triacylglycerol content of the heart, liver and soleus muscle compared to controls. In addition, BVO-treated rats had a decrease in plasma levels of triacylglycerol and total and HDL cholesterol. Plasma fatty acid levels and plasma post-heparin lipolytic activities, such as H-TGL, LPL, T-TGL and MGH were similar to those of control animals fed the standard chow alone.

Heart PDH_a (active portion of pyruvate dehydrogenase) was dramatically decreased in the BVO-fed rats. A faster rate of spontaneous lipolysis was recorded in the isolated perfused preparation of hearts from the experimental animals. The addition of 10^{-7} M of glucagon to the perfusate, however, revealed a lipolytic effect comparable to the one observed in the control rats. In summary, our findings of normal fatty acids and low triacylglycerol plasma levels associated with normal activities of the various PHLA (post-heparin lipolytic activity) enzymes suggest that accumulation of triacylglycerol in heart muscle may not be explained essentially in terms of an elevated uptake and/or increased delivery of plasma fatty acids or plasma triacylglycerol. A decreased in situ catabolism of tissue triacylglycerol also appears unlikely because the spontaneous as well as the glucagon induced lipolysis in the heart both were found to be unimpaired.

Our results suggest that the mechanisms involved in the toxicologic effects of a BVO diet on heart lipid metabolism could be exerted mainly at the level of triacylglycerol biosynthesis rather than a derangement in some known step of their catabolic pathway. Additional studies are necessary to clarify this matter.

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INTRODUCTION

Brominated vegetable oils (BVO) have been used as a food additive for a number of years with the purpose of adjusting the density of essential flavoring oils and to enhance cloud stability in the manufacture of certain citrus-flavored beverages. Relatively little information is available, however, on the toxicological properties of these compounds or of the brominated fatty acids themselves. In this regard, Munro et al (1,2) reported impaired food utilization and growth retardation as well as an enlarged heart, liver and kidney in young male rats fed for 105 days with diets containing 2.5% brominated cottonseed oil. Microscopic examination of the involved tissues revealed lipid accumulation in liver and fatty degeneration involving the entire myocardium. Although less marked, similar changes were observed when the animals were fed with as low as 0.5% BVO.

Only a few biochemical changes associated with BVO ingestion have been documented to the present time. Thus, Munro et al (3) observed reduced liver glucose 6-phosphatase and glucose 6-phosphate dehydrogenase activities after feeding rats with 0.5% brominated cottonseed oil for 120 days. Heart homogenates obtained from rats fed for only 3 days with 40-250 mg/100 g body weight of the above BVO showed a significant decrease in the utilization of palmitic acid. More recently, Jones et al (4,5) reported that lipid bound-bromide was detectable in several tissues of animals given BVO, including heart, liver, kidney, muscle and fat.

Scant information is available presently on basic dynamic biochemical parameters which may be helpful in understanding the mechanisms by which these compounds exert their toxicological effects. The present study, therefore, was aimed at gathering information on lipid metabolism both in vivo and in vitro of heart muscle obtained from rats fed over a period of 105 days with a diet containing 0.5% brominated sunflower-olive oil mixture (BVOD). In this study, measurements of the following parameters were conducted: heart content of triacylglycerol, cholesterol (total and esterified), glycogen and total (PDH) and active (PDH_a) forms of the enzyme pyruvate dehydrogenase; liver content of both triacylglycerol and glycogen

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and serum levels of triacylglycerol, cholesterol and fatty acids. Since the reported increase of triacylglycerol in liver, heart and other extrahepatic tissues also could be the result of a lack of modulation of lipoprotein lipase activities, plasma post-heparin hepatic and extrahepatic triglyceride lipase as well as monoglyceride hydrolyase activities also were investigated. Finally, and in order to observe the dynamic behavior of lipid metabolism under controlled conditions, the effect of glucagon (an agent of known lipolytic effects on heart triacylglycerol) (6-8) was tested in the in vitro perfusion preparation of hearts obtained from BVO-fed animals.

MATERIALS AND METHODS

Diets and Animals

Weanling male Wistar rats weighing 70-90 g were used in all studies. Animals were housed in a controlled temperature (23 C) animal room, with fixed, 24-hr artificial light cycles (12 hr light followed by 12 hr darkness). Initially they were allowed a diet of a standard rat laboratory chow and water. One week after arrival, the animals were divided randomly into two groups. The experimental group received the standard rat laboratory chow (Ralston Purina Company, St. Louis, Missouri, USA) supplemented with 0.5% brominated vegetable oils. The brominated oil was a combination of sunflower and olive oil obtained through a commercial supplier (Abbott, trademark Densitol "A", specific weight 1.305-1.315 at 25 C) and was graded as edible food. Control rats received the above mentioned standard laboratory show supplemented with 0.5% corn oil (STD). Both diets provided approximately 365 calories/100 gm of chow. Food was available ad-libitum, and both groups of rats were maintained on their respective diets for an experimental period of 105 days. The weight of each animal was recorded twice a week throughout the test period. In a separate experiment the caloric intake and weight gain of six animals in each group were assessed twice a week. Caloric intake was estimated as follows: food was weighed before and after remaining in the cage for 24 hr. After separation of feces, weight correction for contamination of urine was carried out by drying the remaining food in an oven, parallel to an appropriate reference sample. Thus, average caloric intake per day per animal (calories/day) was calculated by dividing the total caloric intake per cage by the number of rats housed in it.

On the day of the experiment, food was removed at 7 a.m. unless otherwise indicated, and all experiments were carried out between 10 a.m. and noon.

Perfusion Technique

The hearts of control (n=18) and experimental (n=8) rats were perfused by a modified Langendorff technique. Details of the methodology used have been given elsewhere (8,9). Following perfusion for a 20-min stabilization period, glucagon (1.6 nmol/min) dissolved in Krebs-Henseleit buffer was infused continuously during the next 20 min by means of a Sage syringe pump model 255-L through the injection port directly above the aortic cannula. The effects of glucagon on disappearance of heart triacylglycerol as well as the glycerol output were determined in the same experiment. At the end of the perfusion the heart was quickly frozen with a Wollenberger (10) clamp which had been kept cooled in liquid N_2. The myocardial tissue was then pulverized with a percussion mortar to a fine powder at liquid N_2 temperature. The powder thus obtained was stored in liquid N_2 for up to 1 mo with no detectable changes in triacylglycerol concentration. Glycerol was analyzed at 1-min time intervals on perfusate samples collected from the 18th to 40th min in all experiments. The total net glycerol output attributable to the glucagon effect was calculated by subtracting the baseline levels obtained when the infusion of glucagon was replaced by buffer alone from the total glycerol response observed in the presence of this hormone over the same time intervals in parallel perfusions. In all experiments, the tissue wet wt/dry wt ratios were obtained by drying the perfused hearts or a portion of the frozen heart powder at 125 C for at least 6 hr and reweighing afterwards. The glycerol output and heart triacylglycerol content thus are expressed per gm of dry weight, correcting in this manner for any differences in heart water content.

Assay of Post-Heparin Plasma Triglyceride Lipase and Monoglyceride Hydrolase (MGH)

After i.p. pentobarbital (60 mg/kg body wt), sodium heparin (200 U/kg body wt) was injected into the jugular veins of 10 rats (5 on STD and 5 on BVOD), and blood samples withdrawn 5-7 min afterward from the cava. Post-heparin lipolytic activity (PHLA) as total plasma post-heparin triglyceride lipase activity (T-TGL) was measured by the method of Krauss et al (12). Post-heparin triglyceride lipase activity released from the liver (H-TGL) was determined by inhibition of lipoprotein lipase (LPL) by protamine sulfate (11). We have reported previously (12, 13) that values of H-TGL obtained by heparin sepharose affinity chromatography are comparable to those obtained by inhibition of extra-

hepatic LPL by protamine sulfate, thus validating the use of the latter as a reliable method for measuring post-heparin H-TGL activity. LPL activity was calculated by subtracting the activity found in the assay containing protamine sulfate (H-TGL) from the activity found in the assay containing fresh rat serum as an LPL activator (T-TGL). T-TGL and H-TGL activities were expressed as μmol of glycerol ml^{-1} hr^{-1}. MGH was assayed by a slight modification of the method of Vogel et al (15). More details on all the above enzyme methodologies have been given elsewhere (15,12).

Preparation of Tissue Extracts and Pyruvate Dehydrogenase (PDH) Determinations

Hearts were removed from 18 anesthetized rats (12 on STD and 6 on BVOD) with a Wollenberger clamp precooled in liquid N_2 and the frozen tissue pulverized in a mortar as described above. Pyruvate dehydrogenase activity in crude homogenates was assayed in a coupled reaction system with the final formation of acetylhydroxamate (16). Homogenates of frozen rat heart ventricular muscle ($\cong$ 0.1 g) were prepared at 2-4 C in 0.5 ml of ice cold 20 mM potassium phosphate buffer pH 7.0 containing 40% (v/v) glycerol with a teflon glass homogenizer for 30 sec. Finally, the heart homogenate was sonicated for 4 x 15 sec (with 30 sec pause) at a setting of five with a Sonifier (heat systems, Ultrasonics, Inc.) fitted with a microtip.

Total pyruvate dehydrogenase activity in heart homogenates was assayed after the inactive phosphorylated form present in the homogenate was converted to the active non-phosphorylated form by pre-incubating with 10 mM $MgCl_2$ for 60 min at 25 C (16). For the determination of the active portion of PDH (PDH_a) tissue homogenates were assayed directly. PDH activity can be measured either by following the formation of acetylphosphate and its colorimetric determination as acetyl hydroxamate (16), or by observing the decrease in the spectrophotometric absorbance of p-nitroaniline and the formation of p-nitroacetanilide (17). We have reported previously (18) that values of PDH_a as measured by the formation of either p-nitroacetanilide or acetylhydroxamate are very similar, thus validating the use of the latter as a reliable method for measuring either the active portion or the total pyruvate dehydrogenase activity. A description of the assay and details of the methodology used have been given elsewhere (18).

Analytical

Blood samples obtained from the jugular vein of fed rats (STD n=10; BVOD n=10) anesthetized with pentobarbital (60 mg/kg body wt) were placed immediately in chilled tubes, centrifuged at 4 C and the plasma or serum either used immediately or stored for no longer than three days at -20 C until assayed. Serum triacylglycerol (19), total cholesterol (T_c) (20), high density lipoprotein cholesterol (HDL_c) (20,21) and fatty acids (F.A.) (22) were determined by spectrometric methods. Heart, liver, kidney and epididymal fat pad weights were determined in each group of animals. Heart and liver glycogen was assayed enzymatically as described by Huijing (23) and the results expressed in terms of glucose equivalent from glycogen. Shell fish glycogen yielded equivalent amounts of glucose when analyzed enzymatically (23). Triacylglycerol content of liver, heart and soleus muscle samples was determined on homogenates of frozen tissues powdered by the method of Laurell (19). Tissue DNA content was measured by a micro adaptation of the procedure described by Richard (24). Heart total and esterified cholesterol content were measured in an aliquot of homogenate tissue with acetone:absolute alcohol, 1:1, by the method of Leffler (20).

Total protein was assayed by the method of Lowry, Rosebrough, Furr and Randall (25). Glycerol in the perfusate was determined enzymatically using glycerol dehydrogenase by the fluorometric method of Davidson and Kayala (26). Differences between values obtained in rats fed the standard or the standard supplement with brominated oil diet were tested for significance by the Student's t-test (27).

Triolein, bovine fraction V albumin (essentially fatty acid free) and heparin sodium salt from porcine intestinal mucosa were purchased from Sigma Chemical Company, St. Louis, Missouri. Glucagon was a gift from the Novo Company. Glycerol monooleate 90% was purchased from Calbiochem, San Diego, California. The enzymes and cofactors necessary for the analysis of plasma, tissues and perfusate samples were obtained either from Boehringer Mannheim Biochemicals, Indianapolis, Indiana, or from Sigma Chemical Company, St. Louis, Missouri. All other chemicals were of reagent grade.

RESULTS

Body Weight and Food Intake

Figure 1 shows the weight gain and caloric intake of rats maintained on the STD or BVOD diets for 105 days. As can be seen, the BVOD was accepted readily by the animals; no differences from controls could be observed regarding caloric intake or weight gain. No overall effect on growth could be detected.

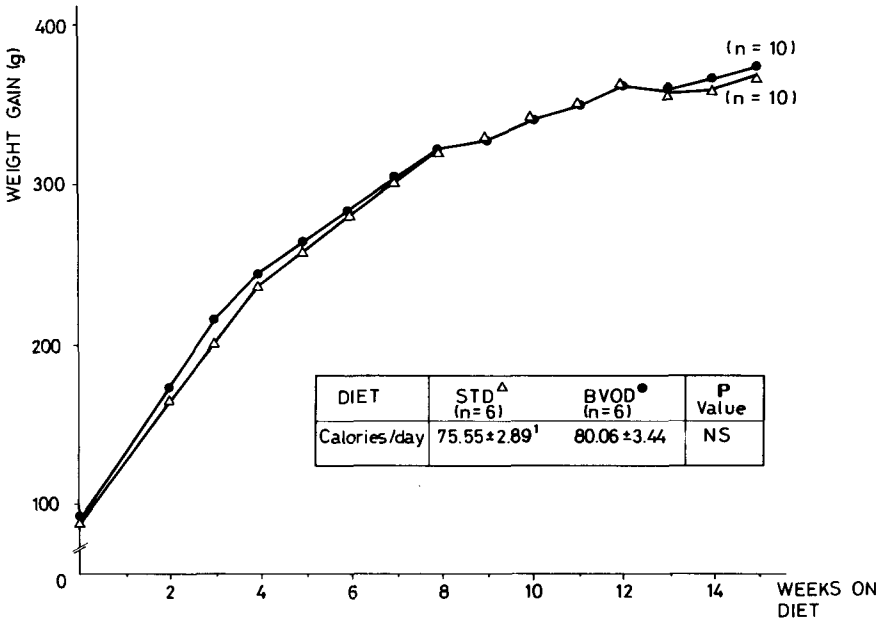

FIG. 1. Body weight and caloric intake of male rats fed brominated sunflower-olive oil at dietary levels of 0% (△) STD or 0.5% (●) BVOD for 105 days. $^1\bar{X} \pm$ SEM. Figures in parentheses indicate the number of rats in each group. NS: nonsignificant. Calories/day indicate average daily caloric intake throughout the experimental period, determined as discussed in Materials and Methods.

Plasma Lipid Profiles

Table 1 shows plasma levels of fatty acids, triacylglycerol, T_c and HDL_c and the ratio HDL_c/T_c in STD and BVOD fed groups. As can be observed, triacylglycerol, T_c and HDL_c levels were significantly lower in the BVOD group, whereas the HDL_c/T_c ratio remained within the range recorded in the STD group.

Weight and Biochemical Tissue Composition of Various Organs

Weight and composition of liver tissue obtained from rats fed the STD and BVOD diets are shown in Table 2. A dramatic increase in triacylglycerol and total protein contents accompanied by a significant increase in liver weight was found in the animals fed the BVOD.

Hearts of BVOD-fed rats showed a significant increase in both triacylglycerol and esterified cholesterol content. Furthermore, heart glycogen was found to be dramatically decreased in these animals, whereas total protein, DNA and heart weight remained comparable to what was found in the control group fed the STD diet (Table 3).

Triacylglycerol content in the soleus muscle (n=5) of BVOD fed rats was elevated: 22.1 ± 3.8 μmol/mg DNA compared to 9.6 ± 2.8 found in controls ($p < 0.01$) (n=8). The kidney relative weight (g/100 g.b.w.) was increased in the BVOD group (0.76 ± 0.06) (n=5) compared to controls (n=5) (0.64 ± 0.01; $p < 0.05$). Epididymal fat pad weight (STD n=5, BVOD n=5), however, remained comparable in both groups of animals.

Pyruvate Dehydrogenase Activity in Heart Muscle

Total pyruvate dehydrogenase activity (PDH) was comparable in both groups of animals (Table 4). The proportion of PDH in the active (PDH_a) form, however, was markedly lower ($p < 0.001$) in animals fed the BVOD diet.

Plasma Post-Heparin H-TGL, LPL, T-TGL and MGH Activities

Plasma post-heparin H-TGL, LPL, T-TGL and MGH activities were similar in both groups of animals as indicated by the following results: STD (n=5), H-TGL: $\bar{X} \pm$ SEM 5.14 ± 0.71; LPL: 1.90 ± 0.56; T-TGL: 7.04 ± 0.54 μmol glycerol ml^{-1} h^{-1} and MGH: 1165 ± 43 μmol glycerol l^{-1} min^{-1}; BVOD (n=5): H-TGL: 5.30 ± 0.44; LPL: 2.65 ± 0.77; T-TGL: 7.96 ± 0.83, and MGH: 1493 ± 99.

TABLE 1

Plasma Lipid Profiles

	Diet		P value
	STD△	BVOD●	
Fatty acid (μEq/l)	349 ± 29[a] (6)	363 ± 119 (6)	NS[b]
Triacylglycerol (m mol/l)	0.55 ± 0.07 (9)	0.29 ± 0.05 (10)	< 0.001
Cholesterol (m mol/l)	1.77 ± 0.12 (6)	1.11 ± 0.08 (6)	< 0.01
HDL cholesterol (m mol/l)	1.16 ± 0.12 (6)	0.63 ± 0.09 (6)	< 0.05
$\frac{\text{HDL cholesterol}}{\text{cholesterol}}$ ratio	53 ± 3 (6)	52 ± 4 (6)	NS

[a] $\bar{X}$ ± SEM. Figures in parentheses indicate the number of rats used for each determination.

[b] NS: nonsignificant.

HDL cholesterol: high density lipoprotein cholesterol.

△ STD: rats fed on the standard laboratory chow supplemented with 0.5% corn oil.

● BVOD: rats fed on the standard laboratory chow supplemented with 0.5% brominated sunflower-olive oil.

TABLE 2

Liver Weight and Biochemical Composition

	Diet		P value
	STD△	BVOD●	
Organ weight/100 g rat	3.47 ± 0.08[a] (6)	4.41 ± 0.07 (6)	< 0.001
DNA (mg/g wet organ)	3.84 ± 0.20 (5)	3.51 ± 0.22 (6)	NS[b]
DNA (mg/100 g rat)	14.0 ± 0.6 (5)	17.7 ± 1.8 (6)	NS
Total protein (mg/100 g rat)	718.7 ± 40.6 (6)	1012.1 ± 32.0 (6)	< 0.001
Glycogen (μmol/100 g rat)	903.3 ± 57.8 (6)	977.3 ± 33.5 (6)	NS
Triacylglycerol (μmol/100 g rat)	34.7 ± 2.8 (8)	80.6 ± 5.2 (8)	< 0.001

[a] $\bar{X}$ ± SEM. Figures in parentheses indicate the number of rats used for each determination.

[b] NS: nonsignificant.

△ STD: rats fed on the standard laboratory chow supplemented with 0.5% corn oil.

● BVOD: rats fed on the standard laboratory chow supplemented with 0.5% brominated sunflower-olive oil.

Effects of Glucagon on the in vitro Perfused Heart Preparation (Table 5)

After a 3-min wash-out period, the triacylglycerol content of the heart was significantly higher ($p < 0.001$) in rats fed the BVOD diet. After 40 min of perfusion with buffer alone, triacylglycerol content fell to comparable levels in both groups of animals. In parallel experiments the addition of glucagon (1.6 nmol/min) from the 20th to 40th minute significantly reduced ($p < 0.05$) the triacylglycerol content to a comparable extent in both groups. The disappearance of heart triacylglycerol during the perfusion was closely paralled by the glycerol output in the perfusate: X ± SEM 6.0 ± 0.5 μmol/g dry weight and 5.6 ± 0.9 μmol/g dry weight, respectively, in the STD-fed rats compared to 5.2 ± 1.7 and 3.8 ± 0.5 in the group of rats fed the BVOD diet.

DISCUSSION

Our results indicate that normal rats readily accepted an experimental diet containing by weight 0.5% of a mixture of brominated sunflower-olive oil, as indicated by a caloric intake and weight gain which were similar to control animals fed the standard chow.

Rats on the experimental diet revealed a significant increase in the triacylglycerol content in the heart, liver and soleus muscle, in contrast to significantly decreased plasma levels of both triacylglycerol and total and HDL cholesterol. Measured plasma post-heparin lipolytic activities such as H-TGL, LPL, T-TGL and MGH remained unchanged. The liver also showed an increase in total protein content and organ weight. The heart exhibited a significant decrease in glycogen content, whereas the organ weight remained normal.

TABLE 3

Heart Weight and Biochemical Composition

	Diet		
	STD[△]	BVOD[•]	P value
Organ weight/100 g rat	0.28 ± 0.06[a] (6)	0.31 ± 0.01 (6)	NS[b]
DNA (mg/g wet organ)	2.20 ± 0.15 (6)	2.21 ± 0.19 (6)	NS
Total protein (mg/100 g rat)	224.2 ± 11.2 (6)	211.6 ± 11.5 (6)	NS
Glycogen (μmol/g wet organ)	15.7 ± 1.2 (8)	6.7 ± 0.7 (8)	< 0.001
Triacylglycerol (μmol/g wet organ)	3.4 ± 0.2 (6)	5.6 ± 0.2 (6)	< 0.001
Total cholesterol (μmol/g wet organ)	5.06 ± 0.18 (6)	6.92 ± 0.31 (6)	< 0.001
Esterified cholesterol (μmol/g wet organ)	1.86 ± 0.21 (6)	4.34 ± 0.28 (6)	< 0.001
$\frac{\text{Esterified cholesterol}}{\text{Total cholesterol}}$ ratio	36 ± 3 (6)	61 ± 2 (6)	< 0.001

[a] $\bar{X}$ ± SEM. Figures in parentheses indicate the number of rats used for each determination.

[b] NS: nonsignificant.

[△] STD: rats fed on the standard laboratory chow supplemented with 0.5% corn oil.

[•] BVOD: rats fed on the standard laboratory chow supplemented with 0.5% brominated sunflower-olive oil.

TABLE 4

Pyruvate Dehydrogenase Activity in Heart of Rats Fed STD or BVOD Diets

	Diet		
	STD[△] (12)	BVOD[•] (6)	P value
Total PDH (U/g wet weight)	5.50 ± 0.32[a]	5.15 ± 0.13	NS[b]
Total PDH (U/mg DNA)	2.33 ± 0.15	2.42 ± 0.19	NS
PDH_a (U/g wet weight)	3.84 ± 0.23	2.32 ± 0.07	< 0.001
PDH_a(U/mg DNA)	1.76 ± 0.17	1.08 ± 0.08	< 0.01
PDH_a (% of total)	71 ± 3	45 ± 2	< 0.001

[a] $\bar{X}$ ± SEM. Figures in parentheses indicate the number of rats in each group.

[b] NS: nonsignificant.

PDH: total pyruvate dehydrogenase activity.

PDH_a: active portion of pyruvate dehydrogenase.

[△] STD: rats fed on the standard laboratory chow supplemented with 0.5% corn oil.

[•] BVOD: rats fed on the standard laboratory chow supplemented with 0.5% brominated sunflower-olive oil.

An increased triacylglycerol content in the heart of rats after ingestion of different types of brominated vegetable oils in concentrations similar to our experimental diet had been described previously by Jones et al (4) and Munro et al (3). The latter authors also reported a lack of palmitic acid oxidation in heart homogenates, which was accompanied by normal utilization of pyruvic acid (3). The conversion of pyruvate to acetyl CoA has been shown to be regulated by the pyruvate dehydrogenase complex, which is itself regulated by a covalent reversible modification (28,29). Moreover, the proportion of PDH in the active form has been shown to be under metabolic and hormonal control in several tissues, the heart muscle among them (29). Our data showed similar values for total PDH activity in the hearts of rats fed the STD or the BVOD diets. PDH in the active form (PDH_a), however, was decreased dramatically in the group of animals fed with the BVOD. It is known that PDH_a levels decrease in starvation due to the fact that an increased provision of fatty acids and ketone bodies as alternate fuels suppresses pyruvate oxidation in heart muscle (30,31). Our animals, however, were not starved and, in addition, showed normal plasma levels of fatty acids. The interconversion of PDH (active to inactive forms) may be influenced by a host of factors, including the $NADH/NAD^+$, acetyl CoA/CoASH and ATP/ADP ratios as well as the pyruvate

TABLE 5

Metabolic Effects of Glucagon in the Perfused Rat Heart Preparation

Diet	Treatment	(n)	Heart triacylglycerol (μmol triacylglycerol/g dry wt) 3 min	20 min	40 min	Glycerol Output[b] 20-40 min (μmol/g dry wt)
STD[△]	None	(9)	19.3 ± 1.2	15.0 ± 0.8	14.2 ± 0.6	
	Glucagon[a]	(9)	– xxx	–	9.0 ± 0.5 x	5.6 ± 0.9
BVOD[•]	None	(4)	36.4 ± 1.0	17.1 ± 0.4	18.9 ± 1.9	
	Glucagon[a]	(4)	–	–	13.8 ± 1.2	3.8 ± 0.5

[a]Infusion of 1.6 nmol/min of glucagon was begun after 20 min of equilibration time and maintained for 20 min up to min 40. Hearts were perfused as described in Materials and Methods with a Krebs-Henseleit medium containing 2.5 mM Ca^{2+}.

[b]Glycerol was analyzed at 1-min intervals in perfusate samples collected from the 20th to 40th minute (for details, see methods). The values given are the mean + SEM. Figures in parentheses indicate the number of experiments.

x: $p < 0.05$, xxx: $p < 0.001$.

[△]STD: rats fed on the standard laboratory chow supplemented with 0.5% corn oil.

[•]BVOD: rats fed on the standard laboratory chow supplemented with 0.5% brominated sunflower-olive oil.

concentration (28,29). The reduction of cardiac palmitate oxidation in animals fed a BVOD could be overcome *in vitro* by the addition of D-L carnitine, ATP and CoASH to the medium (3), the latter two being known to be closely related to the regulation of PDH activity. Finally, there is the possibility that the myocardium of BVOD-fed rats may be hypoxic, as suggested by Munro et al (2), and this could in turn play an important role in PDH activity. Hypoxia has been shown to stimulate glycogenolysis (32), and it is worthwhile to note that we have found an important reduction in cardiac glycogen content in the hearts of BVOD-fed animals.

Lipoprotein lipase activities in post-heparin plasma (mainly from fat and heart origin) are correlated with the removal rate of plasma triacylglycerol, and the activities of these enzymes have been shown to be under hormonal and nutritional control (33,34). Our results showed normal levels of H-TGL, LPL, T-TGL and MGH activities in animals fed the BVOD. Although plasma post-heparin T-TGL activity represents the pool of hepatic and extrahepatic (e.g., cardiac, skeletal muscle, adipose tissue, etc.) enzymes, we were unable to determine the source of the extra hepatic fraction under the present experimental conditions.

The correlation of body weight with serum triacylglycerol has been established previously (35). Moreover, triacylglycerol levels appear to be more closely related to weight gain than to actual weight (36). Our results indicated that, although weight gain and caloric intake were similar in both groups of rats, animals fed the BVOD showed a significant decrease in both plasma triacylglycerol and cholesterol levels. This could be explained by a toxic effect of the bromine-bound lipid on the synthesis, secretion or transport of lipoprotein from the liver (5).

Isolated perfused hearts of BVOD-fed animals showed a faster rate of spontaneous (basal) lipolysis than hearts of rats fed the standard chow (Table 5). The addition of 10^{-7} M glucagon to the perfusate, however, resulted in a comparable lipolytic effect. Similar results were found by Stam et al (37) in triglyceride-enriched hearts obtained from rats fed a trierucate-rich diet. The authors hypothesized that control of lipolysis in the heart may be the result of both the rate of removal of the produced fatty acids and the provision of alternate substrates for energy metabolism. The mechanisms regulating the in vivo lipolysis in animals fed the BVOD are unknown at the present time. Murthy et al (38,39) recently have reported an increase in the activity of glycerolphosphate acyltransferase in triglyceride-enriched hearts of rats made diabetic by alloxan. This increased synthesis of triglycerides coupled with an inhibition of lipolysis by fatty acids and ketone bodies may account for the accumulation of triglycerides under the present experimental conditions. We are unaware of information on the status of the glycerolphosphate acyltransferase in rats fed a BVOD diet.

With regard to synthesis, our data enlarge previous observations in that 0.5% of BVO added to a standard rat chow results in an increased fat content not only in the heart but also in the liver and skeletal muscle. Our findings

of normal fatty acids and low triacylglycerol in plasma, associated with normal activities of the various PHLA enzymes, suggest that the accumulation of triacylglycerol in heart muscle may not be explained in terms of an elevated uptake and/or increased delivery to the tissue of plasma fatty acids or plasma triacylglycerol. Finally, a decrease of the in situ catabolism of tissue triacylglycerol also appears unlikely, because the spontaneous as well as the glucagon induced lipolysis in the perfused heart both were found to be unimpaired.

Our results suggest that mechanisms involved in the toxicologic effect of a BVOD diet on heart lipid metabolism could be exerted mainly at the level of triacylglycerol biosynthesis rather than a derangement in some known step of their catabolic pathway. Further studies are necessary to clarify this.

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Polyphosphoinositides and the Shape of Mammalian Erythrocytes

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ABSTRACT

The relationship between polyphosphoinositide and phosphatidic acid (PA) metabolism and Mg-ATP dependent shape and viscosity changes in erythrocyte ghosts from four mammalian species was examined. Ghosts prepared from rabbit, dog, human and guinea pig erythrocytes were transformed from echinocytes to discocytes within 15 min in the presence of 1 mM Mg-ATP at 25 C. In all species these Mg-ATP shape transformations were associated with a 30-45% decrease in the specific viscosity of the ghost suspensions. Mg-ATP induced a second transformation of discocytic ghosts to cup shape forms without a further decrease in viscosity. A considerable species variation in the rates of Mg-ATP dependent viscosity and shape changes and incorporation of ^{32}P into phosphatidylinositol-4' phosphate (PIP), phosphatidylinositol-4'5'bisphosphate (PIP_2) and especially PA from Mg-[$\gamma^{32}P$]-ATP in ghosts was found. However, the rates of Mg-ATP dependent synthesis of PIP and PIP_2 and shape and viscosity changes in each species were of the same magnitude. Ca^{2+} or neomycin strongly inhibited PIP labeling and Mg-ATP shape and viscosity changes in ghosts of the different species. Ca^{2+} or neomycin usually increased or had little effect on ^{32}P incorporation into PA and PIP_2. The possibility that Mg-ATP-induced changes in erythrocyte membrane shape and deformability are dependent on increases in membrane PIP and PIP_2 is discussed.

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INTRODUCTION

The shape and deformability of erythrocytes is regulated by intracellular ATP and Ca^{2+} (1,2). If erythrocyte ATP levels decrease or intracellular Ca^{2+} concentrations increase, highly deformable discocytic erythrocytes are transformed to echinocytic or spherocytic forms possessing low deformability and high viscosity (2,3). In vivo, the loss of deformability is thought to result in the removal of erythrocytes from the circulation (4). Mg-ATP also can transform isolated echinocytic rabbit or human ghost membranes to discocytes or cup shaped forms (5-7). The Mg-ATP dependent echinocytic-discocytic transformation has been correlated with a decrease in the viscosity of ghost suspensions (5,7). Low concentrations of Ca^{2+} (5,7) or neomycin (5) strongly inhibit Mg-ATP dependent shape transformations or viscosity decreases in rabbit and human ghosts. Ca^{2+} also can convert discocytic human ghosts which were transformed by preincubation with Mg-ATP back to echinocytes (7). It has been postulated that Mg-ATP transforms echinocytic ghosts to discocytes by mechanisms which include phosphorylation of spectrin (8) and phosphatidylinositol to phosphatidylinositol 4-phosphate (PIP) (5). However, a number of workers failed to find a correlation between spectrin phosphorylation and shape in ghosts or in intact erythrocytes (9,10). Quist and Reece (5) suggested that increases in membrane PIP were associated with Mg-ATP dependent viscosity and shape changes because Ca^{2+} or neomycin inhibited both increases in PIP and these conformational changes. Low concentrations of Ca^{2+} 'inhibit' Mg-ATP dependent ^{32}P labeling of PIP in rabbit erythrocyte ghosts by stimulating PIP breakdown by phosphomono- and phosphodiesterase activities (11-14). Neomycin decreases PIP by stimulating Mg kinase mediated phosphorylation of PIP to PIP_2 (15). This study was undertaken to further examine the relationship between Mg-ATP dependent phosphorylation of phospholipids and shape transformations in a number of mammalian erythrocyte membranes. In this study the time courses of Mg-ATP dependent synthesis of PA, PIP and PIP_2 and induced viscosity shape transformations were compared in four different species under identical conditions. The effects of Ca^{2+} and neomycin on viscosity and shape changes and on ^{32}P incorporation into PA, PIP and PIP_2 also were studied.

MATERIALS AND METHODS

Materials

[γ-^{32}P]-ATP was obtained from New England Nuclear, Boston, Massachusetts. Phospholipid standards and neomycin sulfate were purchased from Sigma Chemical Company, St. Louis, Missouri.

Preparation of Erythrocyte Ghosts

Fresh heparinized blood was obtained from rabbits, guinea pigs, dogs and humans of either sex and washed twice with isotonic saline to remove plasma and white cells. Ghosts were prepared by washing packed erythrocytes 2 to 4 times with 8 to 10 volumes of 20 mM Tris HCl, pH 7.6 at 5 C. Ghosts were pelleted by centrifugation for 15 min at 20,000 x g. Packed ghosts were kept at 5 C and used within one hr of preparation.

Determination of Specific Viscosity and Shape

The specific viscosity of suspensions of freshly prepared erythrocyte ghosts was determined using Cannon-Manning Semi-micro viscometers (size 75) as previously described (5,7). The medium usually contained 25 mM imidazole HCl, pH 7.0, 1 mM EGTA, 5 mM $MgCl_2$ and 360 μg/ml of membrane phospholipid. In some experiments, ATP, $CaCl_2$ and neomycin sulfate were included. Shape changes were visualized by phase contrast microscopy at room temperature using a 100X oil immersion objective.

^{32}P Incorporation into Ghost Phospholipids

Ghosts were incubated for various time intervals at 25 C in a final volume of 0.5 ml in 25 mM imidazole HCl, pH 7.0, 5 mM $MgCl_2$, 1 mM [γ-^{32}P]-ATP, 1 mM EGTA and 180 μg of membrane phospholipid. Neomycin or $CaCl_2$ were included in some experiments and free Ca ion concentration was calculated as previously described (5). The reaction was stopped by the addition of 3 ml of cold 5% trichloroacetic acid and 1 mM EDTA. The tubes were centrifuged at 2,000 x g for 10 min and the pellets further washed with 3 ml of H_2O. Lipids were extracted from the pelleted ghosts at 5 C with acidified chloroform:methanol dried under a stream of N_2 as previously described (15). The dried lipid extract was resuspended with 30 μl of $CHCl_3$:CH_3OH:HCl (6:3:0.1) and 10 μl aliquots were either spotted on silica gel 60 plates (E. Merck) or assayed for phospholipid content (13). Thin layer plates were developed in $CHCl_3$:CH_3OH:H_2O:30% NH_3 (45:35:7.5:2.5). ^{32}P labeled phospholipids were located by autoradiography using Kodak OMAT R X-ray film. Labeled phospholipids were scraped from the plates and counted in trisitol. Results are expressed as nmoles of ^{32}P incorporated/mg ghost phospholipid.

RESULTS

Effect of Mg-ATP, Neomycin and Ca^{2+} on Viscosity and Shape

The effects of ATP, Ca^{2+} and neomycin on the specific viscosity of ghosts prepared from four mammalian erythrocyte species were compared under identical conditions. The medium was maintained at a relatively low ionic strength (40 mM) to reduce resealing of the ghosts, especially in the presence of added Ca^{2+}. $MgCl_2$ (5 mM) was present in all studies. Although the ghosts remain leaky under these conditions, they are capable of undergoing discocytic-echinocytic shape transformations. In the absence of added ATP, the viscosity of ghost suspensions prepared from rabbit, human, dog and guinea pig erythrocytes did not change after 25 min incubation at 25 C, and the ghosts remained echinocytic in shape as evidenced by phase contrast microscopy. In the presence of 1 mM ATP, the viscosity of ghost suspensions from all four species decreased 30-45% after 20 min at 25 C (Fig. 1). The times for half maximal change in viscosity were approximately 9, 9.5, 12.5 and 4.5 min for rabbit, human, dog and guinea pig ghosts, respectively. The times required for completion of echinocytic-discocytic transformations concided with times at which the viscosity decreases were maximal. The rate of change in viscosity was at least twice as fast in guinea pig ghosts as in the other three species studied. In guinea pig ghosts, Mg-ATP completely transformed the echinocytic ghosts to discocytes within 7 to 8 min at room temperature. After 12 min incubation, guinea pig ghosts were transformed from discocytes to

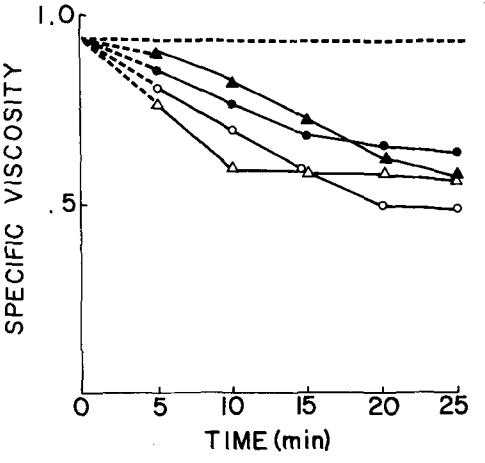

FIG. 1. The effect of Mg-ATP on the viscosity of erythrocyte ghosts from various species. Viscosity was determined in the present of 1 mM ATP in rabbit (●), human (○), dog (▲) and guinea pig (△) ghosts at 25 C. The dotted line represents the viscosity in all four species in the absence of ATP. Results represent the means of at least three separate experiments.

predominantly cup shaped forms. The second transformation of discocytes to predominantly cup shaped forms in the presence of Mg-ATP was not apparent in the other three species until after 30-50 min incubation at 25 C. In the absence of ATP, 10 μM Ca^{2+} had no effect on the shape or viscosity of ghosts of the four species at 25 C. Neomycin (0.3 mM) in the absence of ATP also had no effect on the shape of rabbit, human and dog ghosts but caused guinea pig ghosts to aggregate and precipitate. Ca^{2+} inhibited Mg-ATP dependent viscosity changes by at least 70% in all species (Table 1). Neomycin inhibited Mg-ATP induced viscosity changes 70 to 100% in rabbit, human and dog ghost suspensions. In the presence of neomycin or Ca^{2+} (and ATP), the ghosts of different species remained predominantly echinocytic although the numbers of spicules on the ghosts were reduced and in some cases a small number of discocytes were present.

TABLE 1

Inhibition of Mg-ATP Dependent Viscosity Changes by Ca^{2+} and Neomycin in Erythrocyte Ghosts from Various Species

Species	% Inhibition of Mg-ATP Viscosity Changes	
	10 μM Ca^{2+}	0.5 mM Neomycin
Rabbit	90	100
Human	100	70
Dog	76	76
Guinea pig	70	–

[a]The effects of Ca^{2+} and neomycin on the specific viscosity of ghosts were determined in the presence and absence of 1 mM ATP after 20 min incubation at 25 C. The results for each species represent the mean of 3 separate experiments.

Phosphorylation of Lipids

The time courses of ^{32}P incorporation into PA (Fig. 2), PIP (Fig. 3) and PIP_2 (Fig. 4) from 5 mM $MgCl_2$ and 1 mM[γ-^{32}P]-ATP were determined in rabbit, man, dog and guinea pig ghosts. In agreement with previous studies (18-20), PA, PIP and PIP_2 were the only phospholipids labeled in the presence of Mg-[γ-^{32}P]-ATP. The conditions used here were identical to those used for studying the effects of Mg-ATP on viscosity (Fig. 1) and shape. Under the conditions of these studies, breakdown of labeled PA, PIP and PIP_2 is negligible and therefore ^{32}P incorporation is a measurement of synthesis of these phospholipids (15,16). The rate of ^{32}P incorporation into PA varied by as much as 28-fold in different species. Incorporation of ^{32}P into PA was greatest in rabbit ghosts and was barely

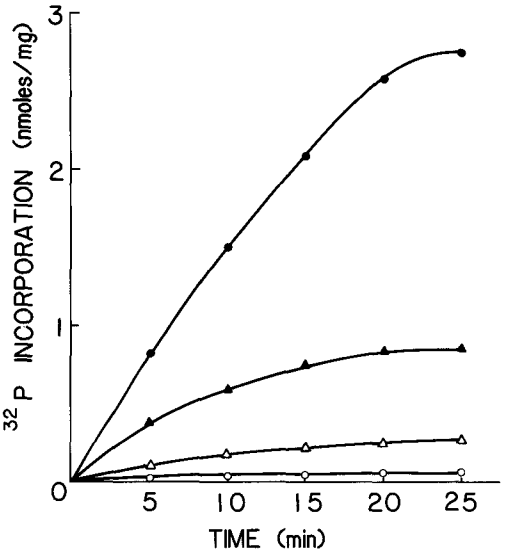

FIG. 2. Time courses of ^{32}P incorporation into phosphatidic acid in erythrocyte ghosts from rabbit (●), human (○), dog (▲) and guinea pig (△) at 25 C in the presence of 1 mM [γ-^{32}P]-ATP. Results represent the means of two to three separate experiments.

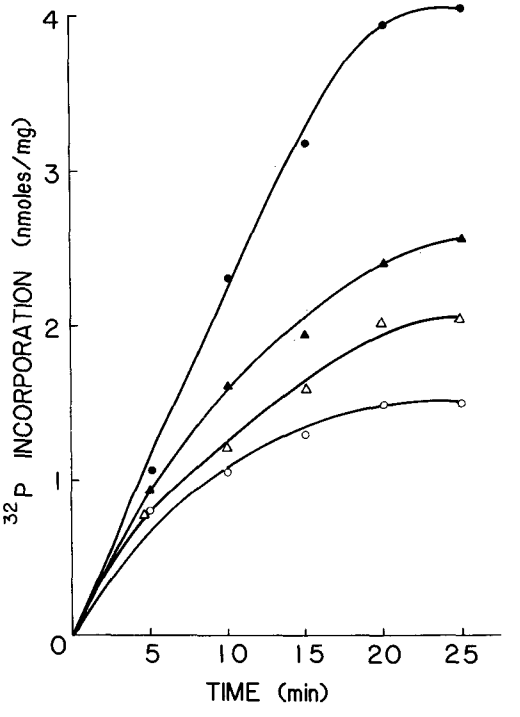

FIG. 3. Time courses of ^{32}P incorporation into PIP in erythrocyte ghosts from rabbit (●), human (○), dog (▲) and guinea pig (△) at 25 C in the present of 1 mM [γ-^{32}P]-ATP. Results represent the means of two to three separate experiments.

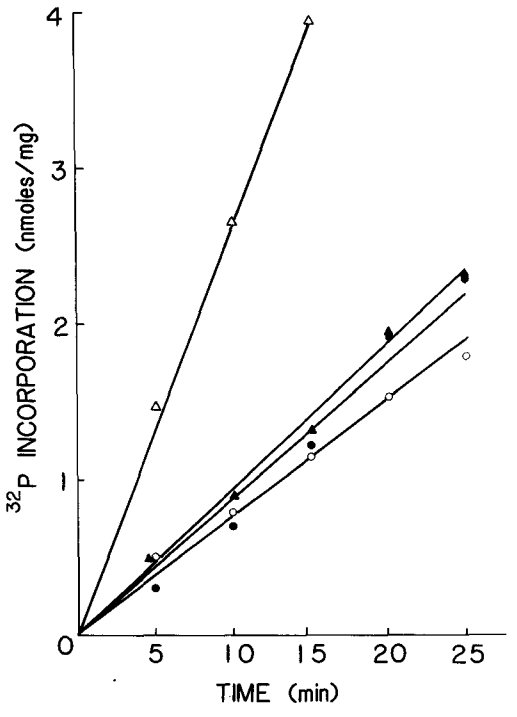

FIG. 4. Time courses of ^{32}P incorporation into PIP_2 in erythrocyte ghosts from rabbit (●), human (○), dog (▲) and guinea pig (△) at 25 C in the presence of 1 mM [γ-^{32}P]-ATP. Results represent the means of two to three separate experiments.

detectable in human ghosts. However, within a given species, variation in ^{32}P incorporation into PA and the other phospholipids was about 25%. Relatively smaller species variations in the rates of labeling of PIP and PIP_2 were found (Fig. 3 and 4). The initial rates of ^{32}P incorporation into PIP were similar after 5 min in all species, but species differences were evident after longer incubation periods. PIP labeling plateaued within 20 min in all species. Incorporation of ^{32}P into PIP_2 was linear in all species for at least 25 min at 25 C (Fig. 4). The rates of ^{32}P incorporation into PIP_2 were similar in rabbit, human and dog ghosts, whereas the rate of ^{32}P incorporation into PIP_2 was approximately three-fold greater in guinea pig ghosts than in the other three species studied.

Effect of Ca^{2+} and Neomycin on Phosphorylation

Ca^{2+} significantly increased ^{32}P labeling of PA in rabbit, human and guinea pig ghosts but had no effect on PA labeling in dog ghosts (Table 2). Ca^{2+} stimulated increases in PA labeling presumably occur secondary to increases in diacylglycerol which results from PIP_2 and

TABLE 2

Effects of Ca^{2+} and Neomycin on ^{32}P Labeling of Phospholipids in Erythrocyte Ghosts from Various Species

Species		^{32}Incorporation (nmoles/mg) PA	PIP	PIP_2
Rabbit	control	2.15	3.25	1.22
	+10 μM Ca^{2+}	4.20	0.74	1.30
	+0.3 mM neomycin	2.25	0.50	9.40
Human	control	0.05	1.30	1.15
	+10 μM Ca^{2+}	0.51	0.48	0.80
	+0.3 mM neomycin	0.05	0.50	4.20
Dog	control	0.72	2.00	1.32
	+10 μM Ca^{2+}	0.75	0.90	1.22
	+0.3 mM neomycin	0.42	0.36	8.85
Guinea pig	control	0.22	1.50	3.95
	+10 μM Ca^{2+}	0.42	0.78	3.81

Ghosts were incubated for 15 min in presence of 1 mM [γ-^{32}P]-ATP, 5 mM $MgCl_2$, 1 mM EGTA, 25 mM imidazole HCl, pH 7.0 ± Ca^{2+} or neomycin. Results represent the means of two to three separate experiments.

PIP_2 phosphodiesterase activities (11). Therefore, lack of Ca^{2+} activation of PA labeling in dog ghosts may suggest that PIP or PIP_2 phosphodiesterase activities are not active in this species under these conditions. Ca^{2+} decreased ^{32}P incorporation into PIP 50 to 75% in all four species and had minimal effects on ^{32}P incorporation into PIP_2. Ca^{2+} inhibition of ^{32}P labeling of PIP is due to stimulation of PIP_2 phosphomonoesterase or phosphodiesterase activities which hydrolyze newly synthesized PIP in human and rabbit ghosts (11-14). Presumably, similar Ca^{2+} stimulated phosphoesterase activities are present in dog and guinea pig ghosts. Under the conditions of these experiments, it is apparent that these phosphoesterase activities do not significantly catalyze PIP_2 breakdown in these species. Neomycin had no effect on PA labeling in human or rabbit ghosts but decreased labeling 42% in dog ghosts (Table 2). Neomycin caused guinea pig ghosts to aggregate and vesiculate. Therefore, the effect of neomycin on phosphorylation of phospholipids in guinea pig ghosts was not examined. Neomycin decreased ^{32}P labeled PIP in rabbit, human and dog ghosts 55-78% and strongly increased ^{32}P labeled PIP_2 in these same ghosts (Table 2) by stimulating kinase mediated phosphorylation of PIP (15).

DISCUSSION

The results of this investigation reveal that Mg-ATP induced shape and viscosity changes in erythrocytes from various mammalian species

by a qualitatively similar mechanism. In rabbit, human, dog and guinea pig ghosts, Mg-ATP induced a similar time dependent decrease in the viscosity of erythrocyte membrane suspensions, although some species variation in the rate of these changes was found. Low concentrations of Ca^{2+} or neomycin strongly inhibited Mg-ATP induced viscosity decreases and echinocytic-discocytic shape transformations in ghosts prepared from these species. It also was found that Mg-ATP induced a second transformation of ghosts from discocytes to cup-shaped forms. This second transformation, however, cannot be detected by a change in viscosity and therefore may not be representative of a change in deformability. Further studies (see below) indicate that the second transformation may be related to an abnormal increase in PIP_2 synthesis in ghosts.

Previously we reported that Mg-ATP-induced viscosity decreases and echinocytic-discocytic shape transformations in rabbit erythrocyte ghosts may be associated with an increase in PIP synthesis (5,15). A comparison of properties of PIP synthesis and Mg-ATP dependent viscosity changes in erythrocyte membranes of four species as reported in the present study support this hypothesis. Although a stoichiometric relationship between the rates of viscosity changes and ^{32}P incorporation into PIP was not apparent, the initial rates of synthesis in all four species were rapid enough to be associated with the conformational changes. The lack of an exact stoichiometric relationship could be explained if different amounts of PIP were required for shape transformations in different species or if changes in other membrane components, such as PIP_2, also were required for these changes to occur. The observations which show that Ca^{2+} or neomycin inhibit Mg-ATP dependent PIP labeling, viscosity decreases and echinocytic-discocytic shape transformations in the four species studied suggest that these Mg-ATP-induced conformational changes may be associated with increases in membrane PIP.

The close similarities between the time courses of Mg-ATP induced viscosity decreases and shape transformations and PIP_2 synthesis in different species further suggest that Mg-ATP induced increases in PIP_2 as well as PIP are required for Mg-ATP dependent membrane transformations. Because neomycin increases PIP_2 and reduced PIP by stimulating the phosphorylation of PIP to PIP_2 (15), changes only in PIP_2 are not sufficient to induce a shape or viscosity change. Thus the relative proportions of PIP and PIP_2 in the membrane could determine membrane shape. As an example of the importance of the ratio of PIP and PIP_2 in shape determination, there also is some correlative evidence that increases in PIP_2 but not PIP may be associated with the second Mg-ATP-induced discocytic-cup shape transformation. For instance, Mg-ATP induced increases in PIP labeling and viscosity changes were complete after 15 min in all species, whereas PIP_2 synthesis continued to increase in parallel to the second shape transformation which occurred after this time. The physiological significance of the second Mg-ATP-induced shape transformation is unclear but it may be an artifact resulting from membrane preparation. Furthermore, it has been shown that rabbit ghost membranes synthesize PIP_2 for at least 100 min (15), which indicates that the ability of isolated membranes to control PIP_2 content may be lost and the production of abnormal amounts of PIP_2 may occur.

A role for PA in Mg-ATP-induced shape and viscosity changes in mammalian ghosts is not supported by the data presented here. For instance, PA labeling was barely detectable in human ghosts and varied by as much as 28-fold in different species. The variation in PA labeling was much greater than the variation in Mg-ATP dependent viscosity changes. Ca^{2+} also tended to increase PA labeling in most species while inhibiting Mg-ATP dependent shape and viscosity changes. Ferrell and Huestis (22) reported that metabolic discotytic-echinocytic transformations may occur secondarily to the degradation of PA and PIP_2 from the inner membrane monolayer. The results of the present study would suggest that a loss of PIP_2 would be necessary only for the transformation to occur.

The mechanism by which changes in PIP and PIP_2 in erythrocyte membranes could lead to membrane conformational changes remains speculative. However, in view of the ability of these highly acidic phospholipids to bind tightly to membrane protein (21), it seems plausible that polyphosphoinositides could modulate the conformation of proteins involved in membrane shape determination. In support of this hypothesis, Sheetz et al (23) reported that PIP_2 can affect the binding of the cytoskeletal protein 4.1 to glycophorin and the lateral mobility of glycophorin in erythrocyte membranes. Polyphosphoinositides also have been shown to be bound to glycophorin isolated from human erythrocytes membranes (24). It is clear that if changes in PIP and PIP_2 can result in major membrane conformational changes, the activities of other membrane components could be affected. Buckley and Hawthorne (20) found that increased phosphorylation of erythrocyte PI resulted in an increase in Ca+Mg-ATPase

activity. Thus, any stimulus which either increases or decreases membrane PIP or PIP_2 could modulate the activity of various membrane components.

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Heated Fat, Vitamin E and Vascular Eicosanoids

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ABSTRACT

A semisynthetic diet containing adequate amounts of vitamin E and 10% (w/w) of a mixture of polyunsaturated oils subjected to heating and characterized by elevated indexes of thermal alteration (polar component, dimer triglyceride, altered triglyceride contents and reduced α-tocopherol levels) was fed to growing male rats for a period of eight weeks. It resulted in a selective alteration of the production of vascular eicosanoids (elevation of platelet thromboxane formation and decrease of vascular prostacyclin release) compared to the values found in rats fed a diet containing a fresh mixture of polyunsaturated oils. Major nutritional parameters, plasma lipids and the fatty acid profiles of plasma, liver and heart lipids were not different in the two groups of animals. Supplementation of an excess vitamin E (300 mg/kg) to the diet containing heated fat neutralized the adverse effects of heated fat on vascular eicosanoid production.
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INTRODUCTION

Thermal oxidation of edible fats is known to alter their nutritional properties, as indicated by several studies in which experimental animals were fed diets containing different levels of oxidized fats for different time periods. Several criteria for assessing toxicity of heated fats have been used (1-3). Work carried out by feeding experimental animals fats that were subjected to severe damage by heat and oxidation has shown the appearance of morphological and biochemical changes in several organs and tissues (4).

A number of newly formed compounds have been detected in heated or fried fat. They were generated through both oxidation and polymerization reactions, especially when the fat was rich in polyunsaturated fatty acids (PUFA) (5,6). However, heating conditions (time, temperature and aeration) and antioxidant levels in the fat modulate the degree of thermal degradation. Heating of dietary fats also results in losses of vitamin E, which is present in appreciable concentrations, especially in highly unsaturated oils. Thus, some of the effects observed after administration of heated fats may depend upon dietary vitamin E deficiency, unless tocopherol levels in the diet are restored.

As increase of the PUFA content of the diet has been recommended by several international medical and nutritional associations because of the beneficial effects of PUFA on plasma cholesterol and arteriosclerosis (7,8). However, the wide utilization of fats which also are highly susceptible to oxidation in cooking and frying may counteract the beneficial effects of the high PUFA content through formation of toxic compounds and/or reduction of dietary vitamin E levels.

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Oxidation products of arachidonic acid have been shown, for instance, to inhibit the enzymes involved in the formation of vascular prostacyclin (9). Similarly, the administration of diets rich in PUFA to rats with low vitamin E levels resulted in enhanced thromboxane formation in stimulated platelets (10) and in reduced formation of prostacyclin like compounds, presumably through enhanced generation of peroxidation products in tissues (10,11). Dietary vitamin E deficiency, by itself, has been shown to increase the balance between platelet thromboxane and arterial prostacyclin production (12).

The aim of our study was to investigate the effects on the balance between the formation of thromboxane by platelets and prostacyclin by arterial walls when growing rats, for eight weeks, were fed diets supplemented with adequate tocopherol levels and containing a mixture of polyunsaturated fats subjected to repeated frying cycles. An additional group of rats was fed a diet which also contained the heated fat but was supplemented with an excess of vitamin E. The effects of the dietary treatments on several nutritional parameters and on plasma and tissue lipid levels and composition also were evaluated. The results indicate that feeding heated fat significantly and selectively altered the balance between thromboxane and prostacyclin production, in spite of the presence of adequate tocopherol levels in the diet. Other parameters of nutritional relevance were not modified. Finally, supplementation of the diet with high levels of vitamin E normalized vascular eicosanoid production.

MATERIALS AND METHODS

Animals

Male rats of the Charles River strain weighing an average of 180 g were used. Four groups of

20 animals each were fed the various diets.

Dietary Treatments

The following four types of diets were fed to four groups of rats: (a) Standard pellet diet (Charles River); Semisynthetic diets supplemented with adequate amounts of vitamin E and containing 10% (w/w) of either (b) a mixture of unsaturated edible oils or (c) the same mixture of oils subjected to repeated frying, and (d) the same diet as (c), supplemented with an excess tocopherol acetate (300 mg/kg of diet). The diets were fed to the animal groups for eight weeks.

Dietary Fats in Experimental Diets

The fat mixture used for the preparation of the semisynthetic diets (10%, w/w) was composed of sunflower seed and palm and hydrogenated peanut oils in the proportion of 55:37:8. The oil mixture was used directly for the preparation of diet (b). For diets (c) and (d), a 10-kg aliquot of the same mixture was subjected to 10 frying cycles of 9 min each at 180 C in the presence of potato sticks in the proportion of 85:15 (oil/potatoes) before the diets were prepared. Diet (d) was obtained by supplementing diet (c) with 300 mg α-tocopherol acetate/kg.

Analysis of Heated Oils

Assessment of the degree of alteration of heated oils was made by evaluating the following parameters: polar component content by column chromatography (13), dimer triglyceride content by gas chromatography (14), the proportion of altered triglyceride in the mixture evaluated as the ratio between a second peak and that of triglyceride after HPLC determination (15) and, finally, the fatty acid composition by GC. The α-tocopherol content in the fresh and heated oils was measured by HPLC on analytic silicic acid columns (16).

Nutritional Parameters

Five rats at random in each group were caged individually, and there remaining 15 animals were subdivided randomly, five per cage. Body weights of both individually caged and group caged animals were measured twice a week and diet consumption was evaluated on a daily basis. At the end of the eight-week period, immediately after the rats were killed, liver, heart, kidneys, spleen and testicles were weighed and subsequently were stored at -20 C for further analysis.

Animal Sacrifice and Preparation of Samples

Animals were killed after eight weeks of dietary treatments. Under light ether anesthesia, blood was drawn through a plastic syringe using Na citrate 3.8% (1/9 of blood) as anticoagulant, from the ascending cava for the preparation of platelet rich plasma and for the determination of plasma lipids. The abdominal aorta was dissected immediately and used for studies of prostacyclin production, whereas liver and heart were used for lipid analysis.

Plasma Lipid Determinations

Plasma was obtained after centrifugation of the citrated blood. Cholesterol and triglyceride concentrations were determined directly by enzymatic procedures, and aliquots of plasma were taken for lipid extraction and subsequent analysis of the fatty acid composition of various lipid classes.

Determinations and Fatty Acid Analysis of Plasma and Tissue Lipids

Total lipids extracted from plasma and from heart ventricles and liver (17) were measured by microgravimetric determinations (18). Analyses of the fatty acid composition of plasma, liver and heart total phospholipids and of liver and heart triglycerides were carried out by gas chromatography of the methyl esters on SP-2330 columns (Supelco, Bellefonte, Pennsylvania), programming temperature (150-210 C, 2.5°/min) of the lipid classes separated by thin layer chromatography.

Preparation of Platelet Rich Plasma (PRP)

PRP and platelet poor plasma (PPP) were prepared by conventional centrifugation techniques. Platelets were counted by contrast phase microscopy, and platelet count in samples for aggregation and thromboxane formation studies was brought to 500,000/μl with autologous PPP.

Platelet Aggregation

The aggregation of platelets in PRP was studied with an aggregometer (ELVI, Logos, Milan, Italy) using increasing concentrations of ADP (2, 4, 8 μM) as aggregating stimulus. Amplitudes of aggregation curves at the various concentrations of the agent were measured.

Thromboxane B_2 (TxB_2) Formation in PRP

TxB_2 synthesis by PRP was evaluated by measuring TxB_2 levels in PRP (500,000 platelets/μl) 2 min after incubation at 37 C in the presence of 5 I.U./ml of thrombin. Reaction

was stopped by addition of 25 volumes of methanol, aliquots were taken and, after evaporation of the solvent, the residue was redissolved in buffer for determination with a specific IRA (19).

Studies of Prostacyclin Release and Antiaggregatory Activity of Isolated Aortas

Synthesis of prostacyclin by isolated aortic segments was evaluated by measuring levels of the stable metabolite 6 keto $PGF_{1\alpha}$ in the fluid perfused through an isolated segment of the abdominal aorta, kept at 30 C in thermostatic bath. PRP was used as perfusion fluid as previously described (20,21), because stimulation of 6 keto $PGF_{1\alpha}$ release and evaluation of the antiaggregatory activity exerted by the vessel wall, measured as degree of inhibition of PRP aggregation after perfusion, are obtained, under these experimental conditions (20). The concentration of 6 keto $PGF_{1\alpha}$ in the perfusate was measured by a specific RIA (22) after extraction with ethyl acetate following acidification, evaporation of solvent and resuspension in appropriate buffer as previously described (20). The antiaggregatory activity of the vessel wall was measured as the increment after perfusion of the threshold concentration of stimulating agent (ADP) required to induce an aggregation curve of about 50% of the maximal, in respect of the concentration required to induce an equivalent response of nonperfused PRP.

RESULTS

Analysis of Diets

The compositions of the reference diet and of the experimental diets are shown in Table 1. The three semisynthetic diets had a slightly lower protein content, an equivalent carbohydrate content and a significantly higher lipid content than the standard diet. The linoleate content as a percentage of energy was twice as high in the experimental diets as in the standard diet, whether the oil mix was fresh or heated.

The parameters which quantify the degree of alteration of the heated fat compared to the fresh fat are shown in Table 2. All the parameters investigated were modified significantly in the heated fats, especially the percentage of triglyceride dimer and the ratio of altered/unaltered triglycerides, which were elevated significantly.

The tocopherol content was reduced remarkably in the heated oil compared to the fresh oil. The vitamin E contents of the various diets are shown in Table 3. Although the heated fat contained only about 10% of the tocopherol present in the fresh oil, the total vitamin E content of

TABLE 1

Composition of Standard and Experimental Diets

Components	Experimental Weight %	Experimental Calorie %	Standard diet Weight %	Standard diet Calorie %
Casein	18	17.8	22.0	24.4
Starch	20	19.8	60.5	67.0
Sucrose	42	41.5	62.3	
Lipid	10	21.7	3.5	8.6
(Linoleate)		7.0		3.7
α-Cellulose	3	—	6.5	
Water soluble vitamin mix[a]	1		7.5	
Lipid soluble vitamin mix[b]	1			
Wesson salt mix[c]	4			
Choline	1			
Total calories	4140		3700	

[a]Thiamine 0.6 mg, riboflavin 1.3 mg, pyridoxine 0.4 mg, niacin 5.0 mg, Ca-pantothenate 4.0 mg, inositol 100 mg, choline chloride 200 mg, liver extract 25 mg, biotine 0.1 mg, PABA 2.5 mg, folic acid 1 mg, vitamin B_{12} 5, cellulose powder up to 1 g.

[b]Vitamin A, 400 I.U.; Vitamin D 100 I.U.; α-tocopherol acetate, 12 mg; menadione 100 μg; cotton oil up to 1 g.

[c]Salt mix (percentage composition) $CaCO_3$ 21.0, $Ca_3(PO_4)_2$ 14.9, KH_2PO_4 31.0, KCl 12.0, NaCl 10.5, $MgSO_4$ 9.0, $MnSO_4$ H_2O 0.02, $FePO_4$ $4H_2O$ 1.47, $CuSO_4$ $5H_2O$ 0.039, KJ 0.005, NaF 0.057, $K_2Al(SO_4)_2$ $24H_2O$ 0.009.

TABLE 2

Characteristics of Fresh and Heated Fat in the Experimental Diets

Indexes of thermal degradation	Fresh	Heated
% Polar compounds	6.60	12.50
% Triglyceride dimers	0.24	0.63
Altered/unaltered triglycerides	0.17	0.63
α-Tocopherol content	460 ppm	40 ppm

TABLE 3

Tocopherol Contents of the Various Diets (mg/kg)

	Through vitamin supplements	In dietary fat	Total
Standard diet, a	—	—	50
Diet containing fresh oil, b	120	46	166
Diet containing heated oil, c	120	4	124
Diet c supplemented with 300 mg/kg α-tocopherol, d	300	4	304

the corresponding diet was more than twice that of the standard diet. The total tocopherol levels in the diets containing either the fresh or the heated oil were similar. The calculated tocopherol/linoleate ratios in the diets were approximately 3.7 mg/g in the standard diet, 6.7 mg/g in the diet containing fresh oil, 5 mg/g in the diet containing heated oil and 12.5 mg/g in the diet supplemented with an excess of vitamin E.

The percentage compositions of fatty acid methyl esters in the lipid fraction of the standard diet and in the oil mixtures are shown in Table 4. The composition of the oils used in the experimental diets differed relative to the lipid fraction in the control diet, in that the levels of 18:0 were 2.5 fold, 18:1 was about 25% higher, 18:2 was one-third lower and linolenic acid was absent.

TABLE 4

Fatty Acid Composition of Fat Extracted from the Standard Diet and of the Oil Mixtures in the Experimental Diets

Acids	Oil mixture in experimental diets		
	Standard diet	Fresh (F)	Heated (H)
16:0	23.4	25.4	25.0
18:0	4.6	10.8	10.4
18:1	25.5	31.0	32.3
18:2 n-6	43.6	32.8	32.3
18:3 n-6	3.0	—	—

General Nutritional Parameters

The growth curves of the various animal groups are shown in Figure 1. The weight increment was uniform in all animal groups, but some differences among groups were observed at various time periods. In fact, the body weights of the group fed the fresh oil mixture were significantly lower than those of the other groups at three, four and five weeks of treatment. The body weights of the group fed the heated fat plus the excess vitamin E supplement were significantly higher than those of the groups fed the fresh and heated fats at seven weeks and than those of all the other groups at the end of treatment.

The average consumptions of diet (weight and Kcal/week) and the caloric efficiencies are presented in Table 5. It appears that the consumption (g/week) of the experimental diets was about 25% lower than that of the reference diet. The average consumptions of the same diets, evaluated as Kcal/week, however, were only about 15% lower than that of the standard diet. This discrepancy was due to the relatively higher caloric content of the experimental diets. The consumption of the diet containing heated fat supplemented with vitamin E was higher than that of the other two experimental diets. The caloric efficiencies (animal weight increment/calories consumed during the dietary treatments) were higher for the experimental diets than for the reference diet.

Weights of several organs measured at the end of dietary treatments are reported both as

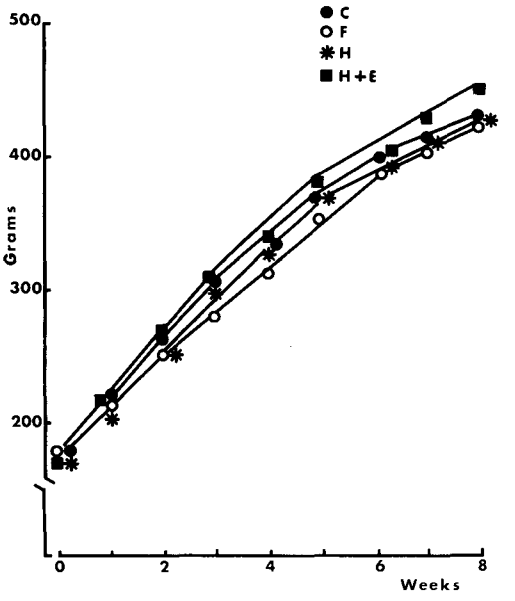

FIG. 1. Body growth curves. C, controls; F, fresh experimental fat; H, heated experimental fat; H + E, heated experimental fat + excess vitamin E (300 mg/kg diet).

TABLE 5

Average Consumption of Diets (weight and Kcal/week) and Average Caloric Efficiency[a]

Dietary groups	Consumed diet		Caloric efficiency[a]
	g/week	Kcal/week	
S	177 ± 3[b]	637 ± 13[b]	41.7 ± 1.5[b]
F	130 ± 8[c]	537 ± 35[b]	49.0 ± 1.7[b]
H	131 ± 4[b]	543 ± 17[c]	50.3 ± 1.1[b]
H + E	139 ± 5[c]	578 ± 21[d]	50.3 ± 3.8[c]

Values are the average ± SD. Values not bearing the same superscript are significantly different from each other ($p<0.005$).

[a]Caloric efficiency = body weight increment/1000 consumed Kcalories during the dietary treatments.

S, standard diet; F, fresh fat; H, heated fat; H + E, heated fat + vitamin E.

TABLE 6

Organ Weights (g and % of body weight)

Dietary groups		Liver	Heart	Spleen	Kidney	Testicles
S	g	16.09 ± 1.49	1.14 ± 0.10	0.70 ± 0.08	2.65 ± 0.23[a]	3.23 ± 0.60
	% bw	3.73 ± 0.16	0.27 ± 0.01	0.16 ± 0.01	0.62 ± 0.04[b]	0.76 ± 0.05
F	g	15.03 ± 1.42	1.18 ± 0.08	0.61 ± 0.10	2.87 ± 0.28	3.30 ± 0.22[b]
	% bw	3.48 ± 0.29	0.27 ± 0.02	0.13 ± 0.02	0.66 ± 0.05	0.78 ± 0.08
H	g	15.07 ± 1.67	1.18 ± 0.08	0.67 ± 0.09	2.97 ± 0.46	3.28 ± 0.31[a]
	% bw	3.39 ± 0.21	0.27 ± 0.01	0.15 ± 0.02	0.67 ± 0.01[b]	0.74 ± 0.07
H + E	g	16.10 ± 2.46	1.24 ± 0.09	0.65 ± 0.08	3.08 ± 0.29[a]	3.60 ± 0.22[a,b]
	% bw	3.47 ± 0.35	0.27 ± 0.02	0.14 ± 0.01	0.67 ± 0.07	0.79 ± 0.05

Values are the average ± SD. Values bearing the same superscript are significantly different from each other ($p < 0.05$). S, standard diet; F, fresh fat; H, heated fat; H + E, heated fat + vitamin E.

TABLE 7

Lipid Content (mg total lipid/g of tissue) of Liver and Heart Ventricles

Dietary groups	Liver	Heart ventricle
S	6.46 ± 0.72	3.35 ± 0.24
F	6.49 ± 0.59	3.25 ± 0.06
H	6.66 ± 1.50	3.18 ± 0.12
H + E	6.68 ± 0.22	3.28 ± 0.13

Values are the average ± SD. S, standard diet; F, fresh fat; H, heated fat; H + E, heated fat + vitamin E.

g and percentage of body weight (Table 6). No major difference was observed among the various experimental groups, except for increments of kidney weights in the groups fed the heated fat (H) and the heated fat + excess vitamin E supplement (H + E) compared to values in the group fed the reference diet, and for increments of testicle weights in the groups fed all the experimental diets compared to values in the group on the reference diet. Lipid contents (mg/g of tissue) in livers and heart ventricles of the various experimental groups are shown in Table 7. No significant difference was observed among the various groups.

Plasma Lipids

Plasma levels of total cholesterol and triglycerides in the various animal groups are shown in Table 8. Cholesterol levels were significantly higher in the groups fed the experimental diets containing fresh fat and heated fat + excess vitamin E compared with values in the reference group, but no difference was observed among the values in the three groups fed the experimental diets. Plasma triglyceride levels were lower in the group fed heated fat than in all the other groups.

TABLE 8

Total Plasma Cholesterol and Triglyceride Levels (mg/100 ml) of Rats Fed the Various Diets

Dietary groups	Cholesterol (mg/100 ml)	Triglycerides (mg/100 ml)
S	60.3 ± 6.4[a,b]	137.0 ± 65.5
F	73.2 ± 7.5[a]	117.6 ± 22.7
H	68.4 ± 13.2	72.2 ± 18.9*
H + E	72.8 ± 16.8[a]	113.6 ± 49.7

Values are the average ± SD. Values bearing the same superscript are significantly different from each other at the following levels: a, $p < 0.05$; b, $p < 0.001$. The triglyceride value bearing an asterisk is significantly different from all the others ($p < 0.001$). S, standard diet; F, fresh fat; H, heated fat; H + E, heated fat + vitamin E.

Plasma and Tissue Fatty Acids

The fatty acid compositions of plasma, liver and heart lipids in the different animal groups are shown in Tables 9, 10, 11, 12 and 13. The fatty acid compositions of plasma phospholipids (Table 9) indicate that in the experimental groups, levels of 20:4 n-6 were about twice as high and those of linoleic acid were lower than in the reference group. Levels of polyunsaturated fatty acids of the n-6 series were not different from each other in the experimental groups.

Fatty acids of liver lipids are shown in Tables 10 and 11. In liver phospholipids (Table 10), levels of 20:4 n-6 were similar in all groups, whereas those of 18:2 n-6 were lower in the groups fed the experimental diets. 22:6 n-3 was detectable only in animals on the reference diet. The total levels of polyunsaturates and the degree of unsaturation were lower in the experimental groups. In liver triglycerides, linoleic acid levels also were lower in the groups fed the

TABLE 9

Fatty Acid Composition of Plasma Phospholipids

Fatty acids	Dietary groups			
	S	F	H	H + E
16:0	32.7 ± 3.2[a,b,c]	27.2 ± 1.9[a]	27.3 ± 0.9[b]	27.0 ± 1.1[c]
18:0	29.7 ± 3.5[a]	30.3 ± 2.2[b,c]	27.5 ± 1.2[a,b]	28.2 ± 1.0[c]
18:1	9.2 ± 1.4[a,b]	10.2 ± 1.7[a,c]	12.5 ± 1.2[b,c]	10.9 ± 1.3
18:2 n-6	19.6 ± 1.3[a,b]	15.5 ± 1.4[a]	16.3 ± 2.4[b]	16.1 ± 2.1
20:4 n-6	8.9 ± 2.3[a,b]	16.8 ± 1.2[a]	17.7 ± 0.3[b]	17.2 ± 0.8
20:4/18:2	0.45	1.08	1.08	1.07

Values are the average ± SD. Values bearing the same superscript are significantly different from each other ($p < 0.005$). S, standard diet; F, fresh fat; H, heated fat; H + E, heated fat + vitamin E.

TABLE 10

Fatty Acid Composition and Unsaturation Levels of Liver Phospholipids

Fatty acids	Dietary groups			
	S	F	H	H + E
16:0	23.7 ± 1.7	26.7 ± 3.2	23.5 ± 1.3	23.3 ± 4.3
18:0	27.7 ± 3.7	31.9 ± 5.1	27.4 ± 3.9	29.4 ± 4.7
18:1	7.4 ± 0.4[a]	10.3 ± 0.8	12.8 ± 1.2	12.9 ± 1.8
18:2 n-6	16.7 ± 2.0[a]	10.1 ± 2.7	12.4 ± 2.8	11.4 ± 2.8
20:4 n-6	21.3 ± 2.7	20.9 ± 5.2	23.9 ± 3.4	23.1 ± 5.8
22:6 n-3	3.1 ± 1.1	tr	tr	tr
20:4/18:2	1.27	2.07	1.93	2.03
Sat	51.4	58.6	50.9	52.7
Mono	7.4	10.3	12.8	12.9
Poly	41.1	31.0	36.3	34.5
U.I.	145	114	133	128

Values are the average ± SD. Sat, Mono, Poly are the sum of the percentage values of total saturated, monounsaturated and polyunsaturated fatty acids. U.I., unsaturation index = sum of percentage of individual fatty acids × number of double bonds. Values bearing a superscript are significantly different from the values in all other groups ($p < 0.005$). S, standard diet; F, fresh fat; H, heated fat; H + E, heated fat + vitamin E.

TABLE 11

Fatty Acid Composition of Liver Triglycerides

Fatty acids	Dietary groups			
	S	F	H	H + E
16:0	37.7 ± 2.4	37.8 ± 3.0	37.5 ± 1.1	38.0 ± 6.8
16:1	3.3 ± 0.7[a,b]	4.2 ± 1.9	6.7 ± 2.2[a]	6.9 ± 2.9[b]
18:0	3.8 ± 0.7	3.3 ± 0.6	3.3 ± 1.2	2.8 ± 0.6
18:1	34.7 ± 3.3	37.3 ± 4.0	38.9 ± 2.4	38.3 ± 1.3
18:2 n-6	23.0 ± 6.6[a,b]	17.5 ± 5.3	13.7 ± 1.6[a]	14.0 ± 6.1[b]

Values are the average ± SD. Values bearing the same superscript are significantly different from each other ($p < 0.05$). S, standard diet; F, fresh fat; H, heated fat; H + E, heated fat + vitamin E.

TABLE 12

Fatty Acid Composition of Heart Phospholipids

Fatty acids	Dietary groups			
	S	F	H	H + E
16:0	17.5 ± 2.8	15.8 ± 1.0	14.7 ± 0.5	14.0 ± 1.3
18:0	25.5 ± 3.4	23.7 ± 1.6	24.5 ± 1.5	24.2 ± 1.8
18:1	10.2 ± 0.5[a]	11.9 ± 1.0[b]	12.4 ± 1.1[b]	11.7 ± 1.5
18:2 n-6	24.6 ± 2.4[a]	19.2 ± 1.7[b]	20.2 ± 2.0[b]	20.5 ± 2.1[b]
20:4 n-6	16.6 ± 2.6[a]	21.1 ± 1.9[b]	19.7 ± 1.5[b]	20.5 ± 0.7[b]
22:4 n-6	–	1.7 ± 0.7	1.7 ± 0.5	2.3 ± 0.8
22:5 n-6	–	5.4 ± 1.9	4.9 ± 1.1	5.6 ± 2.3
22:6 n-3	7.3 ± 3.9[a]	1.2 ± 0.3[b]	1.7 ± 0.5[b]	1.6 ± 0.7[b]
Sat	43.0	39.5	39.2	38.2
Mono	10.2	11.9	12.4	11.7
Poly	48.5	48.6	48.2	
U.I.	170	175	173	181

Values are average ± SD. Values not bearing the same superscript are significantly different from each other ($p < 0.005$). S, standard diet; F, fresh fat; H, heated fat; H + E, heated fat + vitamin E.

TABLE 13

Fatty Acid Composition of Heart Triglycerides

Fatty acids	Dietary groups			
	S	F	H	H + E
16:0	31.7 ± 1.5	28.0 ± 3.4	30.7 ± 0.2	31.3 ± 7.5
16:1	2.6 ± 0.4	3.3 ± 1.1	3.2 ± 0.2	2.9 ± 0.7
18:0	9.2 ± 1.3	8.0 ± 2.2	8.4 ± 1.0	9.0 ± 2.3
18:1	29.1 ± 2.6[a]	40.2 ± 2.9[b]	39.3 ± 2.3[b]	38.4 ± 7.3[b]
18:2 n-6	27.4 ± 1.4[a]	20.7 ± 2.9[b]	18.6 ± 1.4[b]	18.6 ± 3.4[b]

Values are the average ± SD. Values not bearing the same superscript are significantly different ($p < 0.01$). S, standard diet; F, fresh fat; H, heated fat; H + E, heated fat + vitamin E.

experimental diets than in controls.

In heart phospholipids (Table 12), 20:4 n-6 levels were higher and those of 18:2 n-6 were lower in the group on the experimental fats than in the reference group. Also, levels of 22:6 n-3 were remarkably lower in the experimental groups than in animals on the reference diet. The unsaturation levels were similar in all groups. In heart triglycerides (Table 13), 18:1 n-9 levels were higher and those of 18:2 n-6 were lower in the experimental groups than in the reference group.

Platelet Thromboxane Formation

The levels of TxB_2 measured in PRP from the various animal groups at 2 min after thrombin (5 I.U./ml) stimulation are shown in Table 13. Levels were very similar in PRP of the group fed the reference diet and in those fed the experimental diets containing fresh fat or heated fat + excess vitamin E, but in PRP of the group fed the heated fat, values were about twice as high.

Aortic Prostacyclin Production

Levels of 6 keto $PGF_{1\alpha}$, the major stable metabolite of prostacyclin, in PRP from the various groups of animals perfused through isolated segments of aortas, are shown in Table 14. Levels (pg/μl of perfusate) were lowest in the group fed the reference diet. Among the groups fed the experimental diets, the maximal 6 keto $PGF_{1\alpha}$ production was in the group fed the fresh fat and the lowest was in the group fed the heated fat.

Because the experimental diets differed from the reference diet in many respects (protein, lipid, carbohydrate, fiber, etc.), the lower arterial 6 keto $PGF_{1\alpha}$ production observed in the reference group cannot be attributed only to the different lipid content.

The relationships between the release of

TABLE 14

Levels of TxB_2 in PRP after Thrombin Stimulation

Dietary groups	TxB_2 (pg/μl)
S	15.8 ± 3.3[a]
F	19.9 ± 4.4[b]
H	35.1 ± 6.6[a,b,c]
H + E	23.1 ± 5.9[c]

Values are the average ± SE of TxB_2 concentrations (pg/μl) measured in PRP (5×10^5 platelets/μl) 2 min after stimulation with 5 I.U./ml of thrombin.

The values marked with the same letter are significantly different from each other at the following levels: a, $p < 0.01$; b, $p < 0.02$; c, $p < 0.05$. S, standard diet; F, fresh fat; H, heated fat; H + E, heated fat + vitamin E.

TABLE 15

Levels of 6 keto $PGF_{1\alpha}$ in PRP Perfused Through Isolated Segments of Aortas Obtained from the Various Animal Groups

Dietary groups	6 keto $PGF_{1\alpha}$ pg/μl of perfused PRP
S	10.5 ± 1.5
F	18.3 ± 1.1*
H	12.5 ± 1.9
H + E	14.4 ± 0.8

Values are the average ± SE. The value marked with an asterisk is significantly different from the others ($p < 0.02$). S, standard diet; F, fresh fat; H, heated fat; H + E, heated fat + vitamin E.

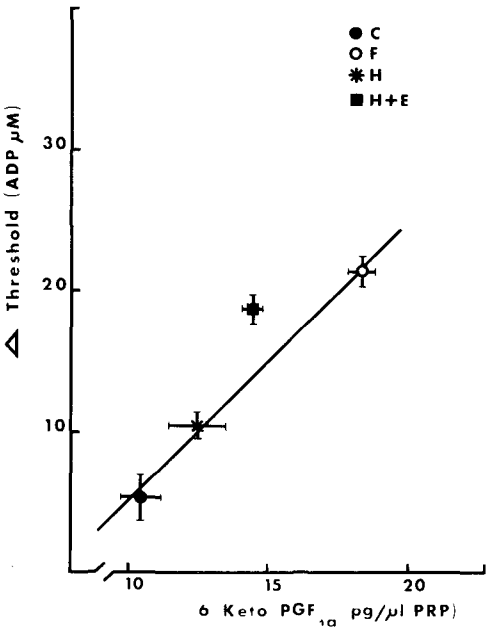

FIG. 2. Relationships between the concentrations of 6 keto $PGF_{1\alpha}$ measured in PRP perfused through isolated segments of thoracic aortas and the increment of threshold concentration (μmolar) for ADP-induced aggregation after perfusion compared with values before perfusion. The line drawn with the exclusion of the H+E point has a correlation coefficient of 1 (y = 15.39+2.01x). C, controls; F, fresh fat; H, heated fat; H+E, heated fat + excess vitamin E (300 mg/kg diet).

6 keto $PGF_{1\alpha}$ by aortas perfused with PRP and the antiaggregatory activity of the arterial wall, measured as an increment of the threshold concentration of the agent (ADP, μM) for PRP aggregation after perfusion, are shown in Figure 2. The release of 6 keto $PGF_{1\alpha}$ and the inhibition of PRP aggregation generally were correlated, but in the group fed the diet containing heated fat + excess vitamin E the inhibition of aggregation was higher than expected on the basis of the concentration of 6 keto $PGF_{1\alpha}$ in the perfusate.

DISCUSSION

Repeated heating of the polyunsaturated fat mixture, although of relatively short duration, markedly modified several parameters in the fat which are considered good indexes of the degree of thermal oxidation and also reduced the vitamin E content. The linoleic acid content of the heated fat, however, was not modified by the heating procedure, indicating that the content of linoleic acid in the fat is less susceptible to modifications by heating than are the other indexes measured.

When the total vitamin E contents of the diets are considered it appears that the level in the heated fat diet was not much lower than in the fresh fat diet. In both diets tocopherol contents were much greater than in the standard diet. In all diets the tocopherol/linoleic acid ratios were higher than 3.7 mg/g, a value definitely greater than 0.6 mg/g, which is considered nutritionally adequate (22).

The parameters describing the nutritional status of the animals fed the experimental diets were not appreciably different from each other, although some difference was observed relative to the group fed the reference diet. Body growth was slightly elevated in the animals fed the diet supplemented with the excess vitamin E over the values for the other groups. In the reference group dietary consumption was higher (both as weight and as caloric intake) than in the experimental groups, as a consequence of the lower caloric content of the standard diet. Caloric efficiency of the reference diet was significantly lower than that of the experimental

diets, presumably also because of its lower fat content.

The fatty acid composition of plasma, liver and heart lipids was identical in the groups fed the experimental diets, regardless of whether the fat was fresh or heated or heated with the addition of excess vitamin E, suggesting that the accumulation of PUFA in tissues was not affected by the administration of thermally altered fat. In the tissues of all experimental groups, levels of linoleic acid were lower than those in the reference group. Levels of arachidonic acid were higher in plasma and heart phospholipids of the experimental groups than in the corresponding fractions of the reference group. In addition, levels of 22:6 n-3 were much lower in heart and liver lipids of the experimental groups. Also, the unsaturation indexes of phospholipids were identical in all animal groups in the heart but not the liver, in which organ higher values in the reference group were calculated. Comparison of the data on the fatty acid compositions of plasma and tissue lipids in the various animal groups indicates that the administration of the experimental diets with a high linoleic acid content and free of PUFA of the n-3 series resulted in a greater conversion of linoleic acid to arachidonic acid which was accumulated in tissues, with a concomitant depletion of the long chain PUFA of the n-3 series, compared to the group on the reference diet.

The plasma cholesterol levels in the experimental group of animals also were very similar, whereas triglycerides were unexpectedly lower in the group fed the diet containing heated fat, compared to the values in the other experimental groups.

Measurement of eicosanoid production in platelets and vessel walls of the animal groups showed interesting differences. Thromboxane production was significantly higher in the group fed the diet containing heated fat than in the other groups.

Several reports in the literature have shown that dietary lipids modulate TxB_2 production by platelets (24,25) and also that a relative vitamin E deficiency results in enhanced platelet TxB_2 and reduced vascular prostacyclin productions (10,12). Since the vitamin E content of the thermally altered fat used in our study appeared to be adequate, the effects on vascular eicosanoid production should be attributed to factors other than vitamin E deficiency in the heated fat fraction.

The observed enhancement of platelet TxB_2 formation in animals fed oxidized fat may be a consequence of altered control of biosynthetic steps, an analogy with the enhanced biosynthetic activity observed in human and experimental hyperlipemia (26,27).

Inhibition by oxidation products of arachidonic acid of the enzymes responsible for prostacyclin formation has been shown by Moncada et al. (10). However, although thermal oxidation of unsaturated fat may generate peroxides, their presence in the heated fat used in our experiment is very unlikely, because these compounds are very unstable.

Prostacyclin release from arterial walls was higher in the groups fed the experimental diets than in the reference group. Among the animal groups fed the experimental diets, the production was much lower in the group fed the heated fat than in that fed the fresh fat; the supplementation of vitamin E tended to restore prostacyclin production, suggesting a protective activity of this treatment, rather than just correction of a deficiency condition.

In the group fed the standard diet and in those fed the fresh or heated fats, a good correlation was observed between the concentration of 6 keto $PGF_{1\alpha}$ in the perfusate and the inhibition of aggregation measured as increment of the threshold concentration of the agent for ADP-induced PRP aggregation after perfusion. It is of interest to observe that the supplementation of vitamin E to the diet containing heated fat potentiated the antiaggregatory activity of the arterial wall, resulting in inhibition of platelet aggregation greater than expected on the basis of the corresponding concentration of prostacyclin in the PRP perfusates.

In conclusion, our data indicate that the administration of dietary fat subjected to relatively mild thermal degradation in the presence of adequate dietary levels of vitamin E selectively alters the balance between the production of TxB_2 in platelets and that of prostacyclin by arterial walls, without modifications of major plasma and tissue lipid parameters. The effects observed after dietary intake of heated fats are of practical interest because a high consumption of fats rich in PUFA, which are oxidized easily and often are used for cooking, is generally recommended for the prevention of cardiovascular diseases. It is also of interest that supplementation of diets containing heated fats with an excess vitamin E normalized the eicosanoid production, providing a means of minimizing the observed toxic effects of thermally oxidized lipids.

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Fatty Acids of Sphingomyelin from Amniotic Fluid of Normal and Diabetic Pregnancies[1,2]

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ABSTRACT

Amniotic fluid collected from 14 normal and 11 diabetic patients was analyzed for phospholipids, and separated sphingomyelin and lecithin fractions were further studied for their fatty acid composition by gas liquid chromatography. Notable differences in percent fatty acid composition of sphingomyelin were observed for palmitic (16:0; diabetic < normal), oleic (18:1; diabetic > normal), behenic (22:0; diabetic < normal) and arachidic acid (20:0; absent in diabetics) in the specimens studied. Notable differences were not observed in fatty acids from lecithin fraction. Fatty acid composition of sphingomyelin from amniotic fluid is similar to fatty acid distribution in sphingomyelin from serum and erythrocyte and suggests maternal origin of the lipid.
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INTRODUCTION

The exclusive source of phosphatidylcholine (PC) and phosphatidylglycerol (PG), the surface active lipids in the alveolar lining, is known to be type II epithelial cells of the lung tissue (1). However, very little is reported regarding the origin of sphingomyelin (SP) in the surfactant. Furthermore, information regarding fatty acid composition of sphingomyelin in the surfactant which may shed some light on its possible origin is severely lacking. This study describes fatty acid composition of sphingomyelin as opposed to that of phosphatidylcholine in amniotic fluid obtained at delivery from normal and diabetic individuals.

MATERIALS AND METHODS

Amniotic fluid was collected from 25 patients admitted to the L.S.U. Obstetrics and Gynecology Service at Charity Hospital in New Orleans. The cases were randomly selected regardless of age or race from normal and diabetic pregnancies. The cases terminated by elective cesarean section in normals and diabetics were due to repeat C-section or breach presentation. Normals were nondiabetic pregnancy patients with no known maternal systemic disease except hypertension. Diabetics were patients diagnosed as diabetics prior to or during pregnancy by means of a glucose tolerance test, some with pre-pregnancy hypertension.

Of the 14 normals, two patients had varying degrees of hypertension which was diagnosed as existing prior to conception. Five of the 11 diabetics were in class A (with two hypertensives), three in class B, one in class C and two in class D_5 (maturity onset with hypertension) according to a recently modified classification (2) (Table 1). Possible growth retardation of the fetus due to pre-pregnant and pre-eclamptic hypertension has been known (3). However, hypertension seems to have no deleterious effect on fetal lung maturity due to its influence on enhanced PC synthesis in fetal lung tissue, to the exclusion of other lung surfactants (4).

To ensure an adequate volume for chemical analysis, 50-100 ml of amniotic fluid was collected at the time of cesarean section. Following an incision through the uterine wall, the membrane was nicked and amniotic fluid was aspirated from the sac with the help of a suction tube and bottle. Specimens contaminated with blood or meconium were discarded. Amniotic fluid was centrifuged immediately for 10 min at 250 × g, and 50 ml of supernatent separated from debris was extracted for lipids by a modified procedure of Folch et al (5).

The extracted lipid was fractionated by one dimensional thin layer chromatography (TLC) on a 20 × 20 cm glass plate precoated with 250μ thick k_5 silica gel, according to a modified method described by Painter (6). Modification involved elimination of cupric chloride pretreatment and diminishing the ammonium content of the developing solvent by one-half. Lecithin, SP and PG fractions were collected

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following their identification by comparing with the standards. To each phospholipid fraction 47.3 μg of heptadecanoic acid (17:0) in heptane was added as an internal standard. Transmethylation of fatty acids in phospholipids fraction was then accomplished by the method of Christie (7), using 2 ml of 0.11 mol/l sulfuric acid in anhydrous methanol at 75 C for 16-18 hr.

TABLE 1

Classification and Distribution of Cases

	Clinical condition	Class	No. of cases	No hypertension	Hypertension prior to conception	Insulin treatment
Normals	No known systemic disease		12			
	Nondiabetic hypertension prior to conception		2			
Diabetics		A	5	3	2	–
		B	3	–	–	+
		C	1	–	–	+
		D_5	2	–	2	+

TABLE 2

Percent of Fatty Acid Methyl Esters of SP with 17:0 Internal Standard[a]

Number	Clinical condition	14:0	16:0	18:0	18:1	18:2	20:0	22:0	22:1	20:4	24:0	24:1
Normal												
6		ND*	25.3	22.4	32.3	8.9	ND	1.0	ND	7.5	2.2	ND
7		ND	24.8	25.0	25.7	5.7	ND	3.2	1.4	9.6	2.9	1.6
8		ND	34.6	13.2	4.8	ND	3.8	9.7	ND	7.3	8.2	15.6
9		ND	22.9	20.9	30.4	6.8	ND	2.6	1.3	8.5	2.0	1.8
10		2.0	33.1	17.0	8.9	ND	4.3	8.5	ND	6.4	8.4	10.2
11		ND	30.8	22.5	12.8	0.8	2.6	7.8	ND	7.5	7.9	7.3
12		1.9	33.7	11.7	5.7	ND	4.7	7.5	ND	3.4	9.2	10.9
18	PP	ND	29.7	26.9	32.8	4.7	ND	ND	ND	4.8	ND	ND
20		1.6	33.7	21.1	24.5	7.7	ND	2.8	ND	7.1	0.8	ND
21		0.7	29.4	21.0	22.0	10.6	ND	1.9	1.8	9.6	1.6	ND
22		ND	28.4	22.0	22.3	9.1	ND	2.2	2.1	9.4	2.2	1.7
23		ND	28.6	26.8	29.2	5.9	ND	ND	ND	4.1	ND	ND
30	HP	ND	39.6	24.7	11.1	1.2	1.4	9.0	ND	1.2	3.2	8.6
31	HP	ND	21.6	22.2	34.9	6.0	ND	3.5	ND	6.7	2.7	2.0
Mean		0.4	29.5	21.0	21.8	4.8	1.2	4.3	0.5	6.8	3.7	4.3
SEM		± 0.2	± 1.3	± 1.1	± 2.7	± 1.0	± 0.4	± 0.9	± 0.2	± 0.7	± 0.9	± 1.4
Diabetic												
13	D_5 (B+HP)	1.6	34.6	18.3	21.0	5.6	ND	4.0	ND	5.7	3.2	4.6
14	B	0.7	21.8	23.0	27.5	5.8	ND	3.2	1.2	7.2	3.4	3.0
15	B	1.2	27.8	19.5	17.9	6.2	ND	5.5	1.2	8.4	4.1	8.4
16	A	1.2	22.5	20.6	30.6	5.1	ND	3.7	1.1	6.6	2.6	2.6
17	A+HP	ND	23.0	21.7	25.3	6.2	ND	3.1	1.4	9.0	3.4	3.7
19	D_5 (B+HP)	0.6	25.1	20.0	29.3	7.1	ND	2.2	1.7	7.4	1.7	1.7
24	A	ND	25.7	28.3	36.4	2.7	ND	0.9	ND	5.1	ND	ND
25	A	ND	27.5	26.6	27.6	7.8	ND	2.9	ND	5.4	ND	ND
26	C	0.9	25.9	24.6	35.3	4.6	ND	0.6	ND	6.5	ND	ND
27	A+HP	ND	33.1	21.6	27.1	5.9	ND	3.9	ND	3.5	3.5	0.9
28	B	ND	23.9	26.6	34.6	5.4	ND	2.1	0.7	5.8	ND	ND
Mean		0.6	26.5	22.8	28.4	5.7	–	2.9	0.7	6.4	2.0	2.3
SEM		± 0.2	± 1.3	± 1.0	± 1.8	± 0.4	–	± 0.4	± 0.2	± 0.5	± 0.5	± 0.8

[a]Fatty acid designated by number of carbon atoms and double bonds.

*ND (none detected) values were assigned to those cases where individual fatty acid comprised less than 0.5% of total.

SEM, standard error of the mean.

PP, placenta previa; HP, hypertension; A, class A diabetic; B, class B diabetic; C, class C diabetic; D_5, hypertension with maturity onset diabetes.

Fatty acid methyl esters were analyzed by gas liquid chromatography (GLC) using a Perkin-Elmer 900 gas chromatograph equipped with a flame ionization detector. A 6 ft, 2 mm diameter glass column packed with 10% SP-2330 Cynosilicone on a 100/200 chromosorb WAW was used for isothermal separation of fatty acid esters at 190 C. The specific retention times and the area of each fatty acid peak (expressed as a percentage of the total) were recorded by the Hewlett Packard 3380 A integrator attached to the chromatographic unit. Fatty acids were identified by comparing the relative retention times of the individual peaks and the standards. The area of each fatty acid peak was corrected based upon the area of the internal standard. Identification of unsaturated fatty acids was confirmed by microhydrogenation procedure using Adam's catalyst (7).

RESULTS

Percent distribution of individual fatty acids in SP and PC from all amniotic fluid specimens are shown in Tables 2 and 3, respectively. It is noteworthy that there is considerable variation in fatty acid distribution in both phospholipids. When fatty acids from normal and diabetic individuals in SP and PC were compared, none of the differences were noted to be statistically significant by Student's t-test. However, in SP notable numerical differences in the mean percentages were observed for palmitic (16:0; diabetic < normal), oleic (18:1; diabetic > normal) and behenic (22:0; diabetic < normal) acids. Arachidic acid (20:0) present in SP only in few normals was consistently absent (undetectable) in specimens from diabetics. Even when nervonic acid (24:1) was present in SP of several speci-

TABLE 3

Percent of Fatty Acid Methyl Esters of Lecithin with 17:0 Internal Standard[a]

Number	14:0	16:0	18:0	18:1	18:2	20:0	22:0	22:1	20:4	24:0	24:1
Normal											
6	2.8	76.8	3.1	8.3	3.8	ND*	ND	ND	4.4	ND	ND
7	3.2	79.9	3.3	6.5	2.2	ND	ND	ND	2.5	ND	ND
8	2.2	69.4	3.8	10.4	7.3	ND	ND	1.2	4.0	ND	ND
9	4.0	74.5	3.2	9.0	3.8	ND	ND	ND	3.6	ND	ND
10	2.7	75.5	3.4	10.2	6.0	ND	ND	ND	1.4	ND	ND
11	4.9	83.7	1.9	5.6	2.7	ND	ND	ND	ND	ND	ND
12	4.4	78.7	2.6	7.7	3.0	ND	ND	ND	2.2	ND	ND
18	4.2	82.2	2.0	6.3	2.9	ND	ND	ND	2.1	ND	ND
20	3.2	76.3	2.8	8.0	5.2	ND	ND	ND	4.0	ND	ND
21	2.7	80.0	3.1	6.3	3.8	ND	ND	ND	3.5	ND	ND
22	2.4	74.1	3.3	8.1	5.4	ND	ND	0.8	5.4	ND	ND
23†	2.4	51.9	1.3	3.9	1.7	ND	ND	ND	1.0	ND	ND
30	ND	49.5	10.3	11.6	16.8	ND	ND	1.7	2.6	ND	ND
31	4.2	76.7	3.2	9.7	3.2	ND	ND	ND	2.6	ND	ND
Mean	3.1	73.9	3.3	8.0	4.8	–	–	0.3	2.8	–	–
SEM	± 0.3	± 2.6	± 0.5	± 0.5	± 0.9	–	–	± 0.1	± 0.4	–	–
Diabetic											
13	4.0	69.2	3.9	10.0	5.7	ND	ND	0.6	4.4	ND	ND
14	3.8	78.4	3.0	7.3	2.5	ND	ND	ND	3.1	ND	ND
15	2.4	62.2	5.4	11.7	8.0	ND	ND	1.2	8.7	ND	ND
16	5.7	77.7	2.4	7.9	2.4	ND	ND	ND	2.5	ND	ND
17	4.4	74.7	3.1	7.9	3.0	ND	ND	ND	4.7	ND	ND
19	3.5	80.6	2.5	6.7	3.3	ND	ND	ND	2.6	ND	ND
24	5.9	76.4	2.2	10.1	2.6	ND	ND	ND	2.2	ND	ND
25	2.3	74.7	3.8	8.1	6.2	ND	ND	ND	2.4	ND	ND
26	3.8	80.2	2.7	7.9	2.6	ND	ND	ND	2.0	ND	ND
27	3.3	82.5	2.2	5.9	2.2	ND	ND	ND	1.2	ND	ND
28	3.9	75.9	3.7	10.0	3.4	ND	ND	ND	2.4	ND	ND
Mean	3.9	75.7	3.2	8.5	3.8	–	–	0.2	3.5	–	–
SEM	± 0.3	± 2.7	± 0.3	± 0.5	± 0.6	–	–	± 0.1	± 0.6	–	–

[a]Fatty acid designated by number of carbon atoms and double bonds.

*ND (none detected) values were assigned to those cases where individual fatty acid comprised less than 0.5% of total.

†Total erroneously low due to chromatographic noise during analysis.

SEM, standard error of the mean.

mens from both groups of patients, many normal individuals showed much higher amounts of this fatty acid than the diabetics (Table 2). Clinical conditions seemed to have no effect on the fatty acid composition of PC (Table 3).

Differences noted in certain fatty acids in amniotic fluid SP of normals and diabetics seem to be much less important than the difference in the distribution of fatty acids in SP from various tissues. It is apparent that in amniotic fluid SP no single fatty acid was predominant (Fig. 1). The three fatty acids, palmitic (16:0), stearic (18:0) and oleic (18:1), were essentially equal in their distribution. This is unlike fatty acid distribution in SP that has been reported in other tissues (Table 4) (8-11).

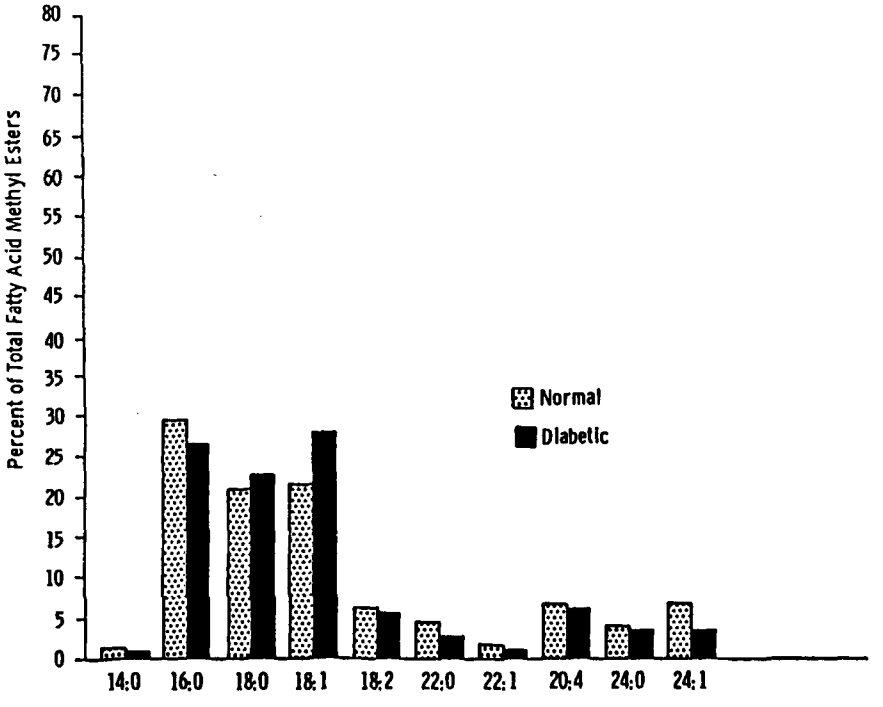

FIG. 1. Mean percentages of fatty acid methyl esters of SP identified by carbon number and number of double bonds for normal and diabetic amniotic fluids.

TABLE 4

Percent Fatty Acid Composition of Lecithin and SP from Different Tissue Sources[a]

	14:0	16:0	18:0	18:1	18:2	20:0	22:0	22:1	20:4	24:0	24:1	Other
Sphingomyelin												
Aorta (9)	–	19.0	12.0	8.0	2.0	10.0	–	–	7.0	14.0	12.0	–
Liver (rat) (11)	–	15.0	13.0	1.0	–	2.0	10.0	1.0	–	25.0	21.0	11.0
Serum (10)	–	33.9	18.3	13.8	21.6	–	–	–	6.7	–	–	4.6
Erythrocyte (17)	1-2	26-28	14-15	11-12	4-5	–	6-7	–	–	12-14	15-17	–
Amniotic fluid (our lab)	0.4	29.5	21.0	21.8	4.8	1.2	4.3	0.5	6.8	3.7	4.3	–
Lecithin												
Aorta (9)	–	21.0	14.0	18.0	7.0	–	–	–	23.0	–	–	–
Serum (10)	–	28.3	21.0	12.6	23.6	–	–	–	10.3	–	–	–
Amniotic fluid (8)	5.1	77.1	4.4	7.1	1.4	–	–	–	–	–	–	–
Amniotic fluid (our lab)	3.1	73.9	3.3	8.0	4.8	–	–	0.3	2.8	–	–	–

[a]Fatty acids designated by number of carbon atoms and double bonds.

DISCUSSION

There is ample evidence for biosynthesis of PC by fetal pulmonary epithelial cells and its timely passage into the amniotic fluid (12), yet very little is known about the origin of SP in amniotic fluid (13). Type II cells do not seem to synthesize SP, although synthesis of sphingolipid and component fatty acids has been shown to occur in other tissues (14,15).

Whatever the origin of SP, it is interesting to note that levels of stearic, oleic, behenic and nervonic acids indicate the tissue source to be metabolically active in interconversion of fatty acids (16). Due to the small number of cases studied, the observation that some fatty acids in diabetics were different from those in normals may have no statistical significance. Due to several biological variables, even with a large number of cases in study, the difference may prove to be of a minor degree. Whether such small differences in distribution are suggestive of the influence of maternal condition on the formation and conversion of fatty acids in SP may be interesting to study. In many respects, though, major fatty acids of SP from amniotic fluid resemble those in other tissues (Table 4) (17). Fatty acids in PC from amniotic fluid, on the other hand, clearly indicate a tissue source independent of maternal blood (18-20).

In conclusion, fatty acid distribution of SP of amniotic fluid suggests a maternal rather than fetopulmonary source for this phospholipid.

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The Di- and Triesters of the Lipids of Steer and Human Meibomian Glands

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ABSTRACT

Three groups of diesters have been isolated and identified in the lipids of steer meibomian glands. The first group, designated as αType I, with the abbreviated formula FA-αOHFA-FAlc, consisted of α-hydroxy fatty acids esterified to fatty acids and fatty alcohols in the approximate molar ratio 1:1:1. The second group, designated as ωType I-St, with the abbreviated formula FA-ωOHFA-St, consisted of ω-hydroxy fatty acids esterified to fatty acids and sterols in the approximate molar ratio 1:1:1. The third group, designated as α,ωType II, with the abbreviated formula FA-α,ωdiol-FA, consisted of α,ω-diols esterified to 2 moles of fatty acids. The sum of the different diesters comprised about 9% of total steer meibomian lipids.

Capillary GLC of the fatty acids of αType I diesters showed the fatty acids to be a family with a two-cluster profile, one at C_{12} to C_{20} and the other at C_{21} to C_{31}, with anteiso chains predominating. Fatty acids from ωType I-St and α,ωType II diesters gave mainly a one-cluster profile in the short chain region with prominent anteiso and $C_{18:1}$ peaks. Fatty alcohols of αType I diesters were mainly long chain, C_{23} to C_{30}, with anteiso chains predominating, while the α-hydroxy fatty acids were short chain C_{13} to C_{18} acids with C_{16} predominating. The sterols in diesters ωType I-St were cholesterol (~60%), Δ7 cholestenol (~35%) and an unidentified compound (~5%) with a GLC retention time slightly longer than Δ7 cholestenol on SE-30 phase. The ω-hydroxy fatty acids and α,ω-diols both were of exceedingly long chain lengths, C_{29}-C_{38}, and showed similar GLC profiles. Two types of triesters comprising approximately 1% of total steer meibomian lipids have been isolated but incompletely characterized. In terms of molar ratios, one group of triesters gave fatty acids:ω-hydroxy fatty acids:α-hydroxy fatty acids:sterols + fatty alcohols as approximately 1:1:1:1. The other contained fatty acids, α-hydroxy fatty acids and α,ω-diols in what appears to be a complex mixture of several triesters. Diesters ωType I and α,ωType II also were found in human meibum. Hitherto these two diesters have not been found in any animal tissue.

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INTRODUCTION

Within the eyelid of animals there is a row of a large sebaceous type of gland, the meibomian gland, which excretes an oily, waxy substance onto the edge of the eyelid. Some of this excreta then spreads out on the wet surface of the cornea, forming the lipid layer of what is known as the preocular tear film.

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Abbreviations: FA = fatty acid; FAlc = fatty alcohol; St = sterol; Chol = cholesterol; FAME = fatty acid methyl ester(s); SE = sterol ester(s); WE = wax ester(s); TG = triacyl glycerol(s); C = chloroform; M = methanol; HAc = acetic acid; TLC = thin layer chromatography; ANS = aniline naphthalene sulfonic acid; GLC = gas liquid chromatography.

In the literature, diesters of α-hydroxy fatty acids have been designated as Type I diesters and diesters of 1,2-diols as Type II diesters. In this paper we wish to generalize the term "Type I" diesters to include diesters of all hydroxy fatty acids no matter what position the hydroxyl group occupies. We also wish to extend the term Type II diesters to include diesters of any long chain diol regardless of the location of the two OH groups. When we want to be more specific as to the location of the OH groups for both types of diesters, we will use a prefix of a Greek letter(s) or Arabic numeral(s). To specify the alcoholic moiety of a di or triester, we will use the suffix FAlc to indicate fatty alcohols or St to indicate sterols.

Triesters will be similarly defined. Triesters Type I will be those containing only hydroxy fatty acids and Type II will be those containing a long chain diol plus a hydroxy fatty acid. We also wish to abbreviate structural formulas using a hyphen or line to signify an ester linkage. The following examples illustrate the usage:

diesters Type I — αType I = FA-α OHFA-FAlc; ωType I-ST = FA-ωOHFA-St

diesters Type II — α,βType II = FA-α,βdiol-FA′ or FA-α,βdiol FA′; 2,3Type II = FA-2,3diol-FA′ or FA-2,3diol FA′; α,ωType II = FA-α,ωdiol-FA′ or FA-α,ωdiol FA′

triesters Type I — FA-αOHFA-ωOHFA-St; FA-ωOHFA-αOHFA-FAlc

triesters Type II — FA-αOHFA-α,ωdiol-FA′; FA-ωOHFA-1,2diol-FA′

Although the meibomian gland is a sebaceous type of gland, its excreta, meibum, differs markedly in chemical composition from sebum, the excreta of the ordinary sebaceous gland. Meibomian lipids have a variety of special functions: They conserve tears by decreasing water evaporation from the tear film; they prevent eyelid skin from becoming overly wet and swelling, and they seal the lids during sleep, thus preventing the corneal surface from a damaging drying out.

In earlier work we have identified about 75% of the meibomian lipids of steer and human which were found to be remarkably similar (1). These lipids consist mainly of sterol esters (~30%), wax esters (~35%) and minor amounts of hydrocarbons, triacylglycerols, unesterified fatty acids and free cholesterol totalling about 10%. Approximately half of the remaining unidentified 25% are components migrating on silicic acid TLC into what we are calling the "diester region." This region lies between the nonpolar monoester region (i.e. where sterol esters and wax esters migrate) and the triester region (i.e. where triacylglycerols migrate). Borders of all these regions are not exact, and overlapping of regions due to differences in chain length, unsaturation and branching of the fatty chains can occur.

The saponified products of the total meibomian lipids from both the steer and the human yielded three lipid components, each with two esterifiable groups, i.e. OH or CO_2H. These difunctional group lipid components were α-hydroxy fatty acids (2), ω-hydroxy fatty acids (3) and long chain α,ω-diols (4). We have some preliminary evidence that still a fourth difunctional group lipid component is present in very small amounts in both the steer and human meibomian lipids, namely α,β-diols. If the hydroxyl group(s) in each of these substances esterifies with a fatty acid, and the carboxyl group esterifies with a fatty alcohol or sterol, we have a diester. If any of these esterifications take place with a second difunctional group lipid component, and the remaining hydroxyl or carboxyl groups esterify with a simple fatty acid, fatty alcohol or sterol, we would then have a triester. In like manner the possibility exists for the formation of tetraesters, pentaesters, etc. Partial hydrolysis of the diesters also could occur, yielding polar monoesters, the polar moiety being either an OH or a CO_2H group, depending upon whether an acid or an alcohol was removed. Similarly, partial hydrolysis of triesters could yield two polar monoesters or a polar diester plus a fatty acid or a fatty alcohol or a sterol.

Diesters of Types I and II (see Abbreviations) have been found in the skin surface lipids of many animals (5-10), in vernix caseosa (10-12) and in the preen glands of many birds (13,14). Triesters also have been found in the skin surface lipids of the rhino mouse (15,16) and the cow (10) and in beeswax (17). The present report describes the identification of three groups of diesters in meibomian lipids, here designated as αType I, ωType I-St and α,ωType II. To the best of our knowledge, this is the first report on the occurrence of the latter two diesters in any animal tissue. A complex group of triesters also has been incompletely characterized. A preliminary report of some of these findings has been made (18).

MATERIALS AND METHODS

In this study, lipids were obtained from two sources from the steer: from whole glands and from excreta (meibum). Lipids from both steer sources and meibum from humans were obtained as previously described (1). TLC of the lipids of both sources from the steer showed a great similarity. Furthermore, TLC of the steer lipids also were quite similar to the human lipids, although small differences, mainly in spot intensity, did exist. To distinguish between these two sources of lipids we shall call lipids derived from total meibomian glands "meibomian lipids," and lipids derived from excreta "meibum lipids." The latter are obtained by expressing the eyelids.

Dry column chromatography according to Loev et al. (19,20) was used to separate 768 mg of crude steer meibomian lipids. A nylon column 3.1 cm × 50 cm containing 258 g silica gel, Woelm (Waters Associates, Inc., Framingham, Massachusetts) was developed with benzene, then sectioned from the bottom upward into one piece 5 cm in length followed by 15 successive pieces 3 cm in length. Lipid from each successive section (designated here as Fractions Nos. 1 to 16, of our Column 210) was obtained by extracting the silica gel with benzene followed by a series of extractions with C/M (9:1, v/v) until negligible amounts of lipid were recovered.

Lipid from Fractions Nos. 1 to 4 weighed 487, 30.0, 4.6 and 1.1 mg, respectively, and TLC on SiO_2 of all fractions showed that only Nos. 1 to 4 had material migrating in the diester region. The bulk of Fraction No. 1, i.e. 480 mg, was then rechromatographed on another column of 52 g SiO_2, 100-200 mesh (Clarkson Chemical Co., Williamsport, Pennsylvania) with dimensions 2.3 cm × 22 cm (designated here as our Column 211). Hexane

eluted 2.7 mg of hydrocarbons (Fraction No. 1), and 20% benzene in hexane eluted 391.7 mg of sterol esters plus wax esters (Fractions Nos. 2 to 8 plus the bulk of No. 9). Since TLC showed no diesters in these fractions, except for a small amount in No. 9, nothing further was done with them. Fractions Nos. 10 through 24, eluted mainly with 40% benzene in hexane, contained the di- and triesters of this study. Fractions Nos. 25 and 26 (15.3 mg), eluted with benzene, and Fraction No. 27 (10.4 mg), eluted with 10% M in C, apparently were decomposition products as judged by the long tailing spots in TLC. Figure 1 shows the TLC of representative Column 211 Fractions.

To isolate and identify the di- and triesters, we used preparative TLC on Whatman LK5D plates (Whatman, Inc., Clifton, New Jersey) developed in hexane/benzene (50:50, v/v). These plates have a preadsorbent or leader portion on which the lipid is spotted. This allows the spotted lipid, during early development, to start as a narrow band when it reaches the adsorbent at the leader edge, thus enhancing resolution. We also used preparative TLC on $Mg(OH)_2$ plates, developed in 1.5 to 3% ethyl acetate in hexane, to separate esters containing sterols from those containing fatty alcohols. This separation will be discussed later. Components were made visible by spraying with ANS, a nondestructive spray, and viewing under UV. They were scraped off the plate and extracted with chloroform. Saponifications were carried out in test tubes with Teflon-lined screw caps, by heating the lipid for 3 hr at 90 C with a 20-fold excess of 10% KOH dissolved in ethanol/water (9:1, v/v). After the saponified products were acidified with HCl and extracted with

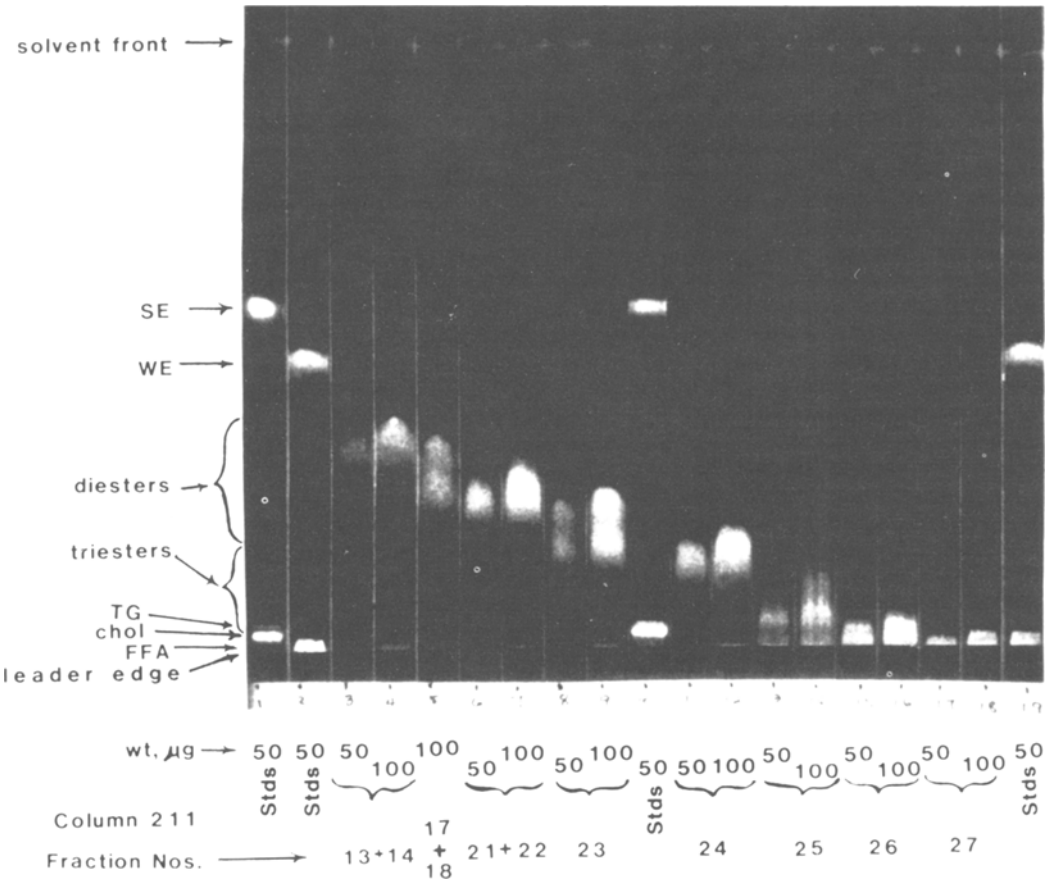

FIG. 1. TLC on SiO_2 of representative Column 211 fractions. Plate was developed with hexane/benzene 35:65, v/v. This plate and all subsequent SiO_2 plates were 250µ thick 20 cm × 20 cm Whatman LK5D (if 19-channel) or 5 cm × 20 cm Quanta/Gram LQD (if 4-channel) plates. ANS spray was used throughout for visualization under UV.

chloroform, they were subjected to TLC on a Quanta/Gram LQD four channel plate (Whatman, Inc.) developed linearly in 4 stages: first solvent C/M/water/HAc (90:10:0.9:0.3, v/v/v/v) to 2 cm above the leader edge, then dried 10 min on a hot plate at 55 C; second solvent C/M (96:4, v/v) to 7 cm above the leader edge, then dried 5 min at 55 C; third solvent C/M/conc NH_4OH (97:3:0.3, v/v/v), to 11 cm, then dried at 55 C for 5 min; fourth solvent, benzene to the score line at 14 cm above the leader edge. All plates were prewashed continuously overnight with ethyl acetate by placing the plate so that it protruded slightly out of the chamber, and placing the chamber cover against the plate so that when the solvent reached the point of contact of the cover it could evaporate into the hood, thus creating a continuous wash throughout the night.

Cholesterol and spots above cholesterol were scraped off the plate and extracted 3 times with chloroform. All remaining spots were extracted with C/M (2:1, v/v). All extracts were filtered through a Pyrex #36060 4.5-5.5 fine sintered glass filter, and the solvents were blown off with prepurified nitrogen. The lipid was then gently extracted with hexane, leaving behind in the flask any precipitated SiO_2. All lipid fractions then were weighed to 0.1 μg on a Cahn 25 Automatic Electrobalance (Ventron Corp., Cahn Instruments Div., Cerritos, California) except the different types of fatty acids, which first were converted to the methyl esters (21) before weighing. Weights of SiO_2 blanks taken from plates put through the 4-stage development system described above, then sprayed with ANS, rarely exceeded 1 or 2 μg. To calculate molar ratios, an average molecular weight for each lipid class was estimated from their GLC patterns. These estimated average molecular weights were 382, 386, 475, 277, 510 and 286 for the FAlcs, sterols, α,ω-diols, FAMEs, ωOHFAMEs and αOH FAMEs, respectively. A 5% correction was made to account for the increase in weight due to addition of the methyl group in the methyl esters of the fatty acids or α-hyroxy fatty acids; the corresponding methyl group correction made for the higher molecular weight ω-hydroxy fatty acids was 2.8%. Fatty alcohols and diols were acetylated by Farquhar's method (22). GLC was performed on a Varian 3700 instrument equipped with a flame ionization detector and an on-column injector (J & W Scientific Inc., Rancho Cordova, California) using a fused silica capillary column, 25 m × 0.25 mm, with chemically bonded SE-30 phase, 0.4μ film thickness (Chromapon Inc., Whittier, California). Temperature programming was from 85 C to 325 C at 7°/min with helium the carrier gas at a flow rate of 1.8 ml/min.

GC-MS was performed as previously described (3).

RESULTS AND DISCUSSION

Table 1 shows the portion of Column 211 which contained the diesters and triesters of steer meibomian lipids. These appeared in Fractions Nos. 9 through 24 (TLC of Fractions Nos. 9 through 12 are shown in Figure 2 and that of representative later eluting fractions are shown in Figure 1). Present were diesters α-Type I, ωType I and α,ωType II plus a mixture of triesters Type I and Type II. Before describing the isolation and identification of individual classes of diesters and triesters, we wish to comment on the general chromatographic behavior of these closely migrating substances on silicic acid. Referring to Figure 3, note that sterol esters (with one ester linkage) generally would have greater R_f values than wax esters (also with one ester linkage), and these classes are nearly completely separated from each other (compare a with b). Next, note that diesters of α-hydroxy fatty acids have greater R_f values than diesters of ω-hydroxy fatty acids of comparable chain lengths and unsaturation (compare c with e). Presumably, adsorptive sites are not as readily available for the two closely spaced ester groups of the diesters of α-hydroxy acids as they are for the widely separated ester groups of diesters of ω-hydroxy acids. But note again that when one of the ester groups of the ω-hydroxy fatty acid is with a sterol rather than a wax alcohol, again the diester containing the sterol migrates farther than one containing a wax alcohol (i.e. compare d with e). Note that e would have the same R_f as f, if all corresponding chains have the same length and degree of unsaturation, because the only difference in structure would be the direction of the ester linkage. The triesters (with three ester linkages) would be expected to adsorb to SiO_2 more strongly than the diesters with only two ester linkages, but here again one would expect ester linkages with sterols to be more weakly adsorbed than those with wax alcohols. Differences in chain length and unsaturation could further complicate the issue in that longer chain lengths would increase migration slightly while more unsaturation would decrease it slightly.

Diesters αType I FA-αOHFA-FAlc

Diesters αType I, the first of the diesters to elute from Column 211, were found mainly in

TABLE 1

Portion of SiO_2 Column Chromatogram of Total Steer Meibomian Lipids Containing Diesters and Triesters

Col. 211[a] Frac. No.	Wt. mg		Wt. of diesters αType I mg	Wt. of diesters ωType I-St mg	Wt. of diesters α,ωType II mg	Wt. of remaining unidentified "diesters"	Wt. of triesters[d] mg
9	7.80[b]		0.15				
10	1.99		1.11				
11	2.46		2.41				
12	3.76		3.61	0.12			
13	3.28	8.68	0.87	7.38	0.43		
14	5.40						
15	5.20	11.09		7.79	2.00	1.30	
16	5.89						
17	4.44	8.80		6.16	2.11	0.53	
18	4.36						
19	3.45	6.56		3.29	2.62	0.65	
20	3.11						
21	5.94	8.66		3.63	4.28	0.75	
22	2.72						
23	3.73				0.56		3.17
24	2.80						2.80
Total	66.3		8.2	28.4	12.0	3.2	6.0
Mg estimated from Fr. 2 & 3 Col. 210			1.0	7.0	5.0		2.0
Grand total			9.2	35.4	17.0	3.2	8.0
% of total steer meibomian lipids[c]			1.3	5.0	2.3	0.4	1.1

[a]These fractions were eluted mainly with 40% benzene in hexane. TLC shown in Figs. 1 and 2. Other fractions were eluted as described in Materials and Methods.

[b]The remainder of Fractions 9 through 12 were sterol and wax esters.

[c]Calculated on the basis of 725 mg total meibomian lipids recovered from Col. 210.

[d]Additional triesters may be present below the diester region. See text.

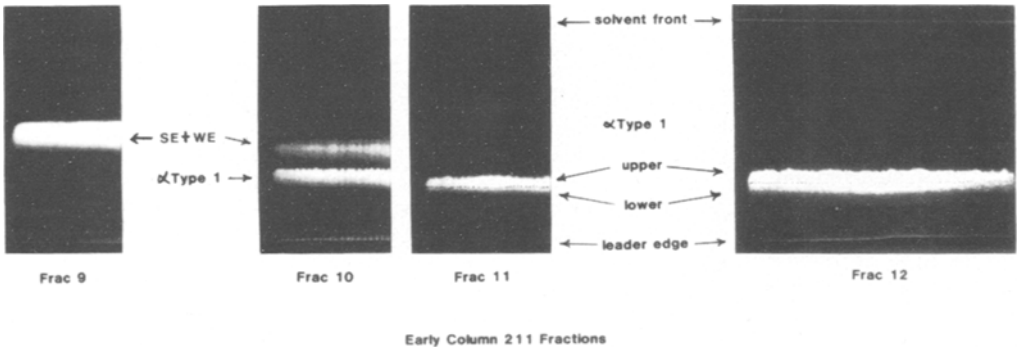

FIG. 2. Preparative TLC on SiO_2 of Column 211 Fraction Nos. 9-12. Plates were developed with hexane/benzene 50:50, v/v. Type of plate and spray used as in Fig. 1. Samples applied were: Frac. 9, 3.1 mg; Frac. 10, 1.2 mg; Frac. 11, 1.6 mg and Frac. 12, 3.0 mg. Plates show separation of αType I diesters from SE and WE and its purity from contamination with ωType I-St diesters.

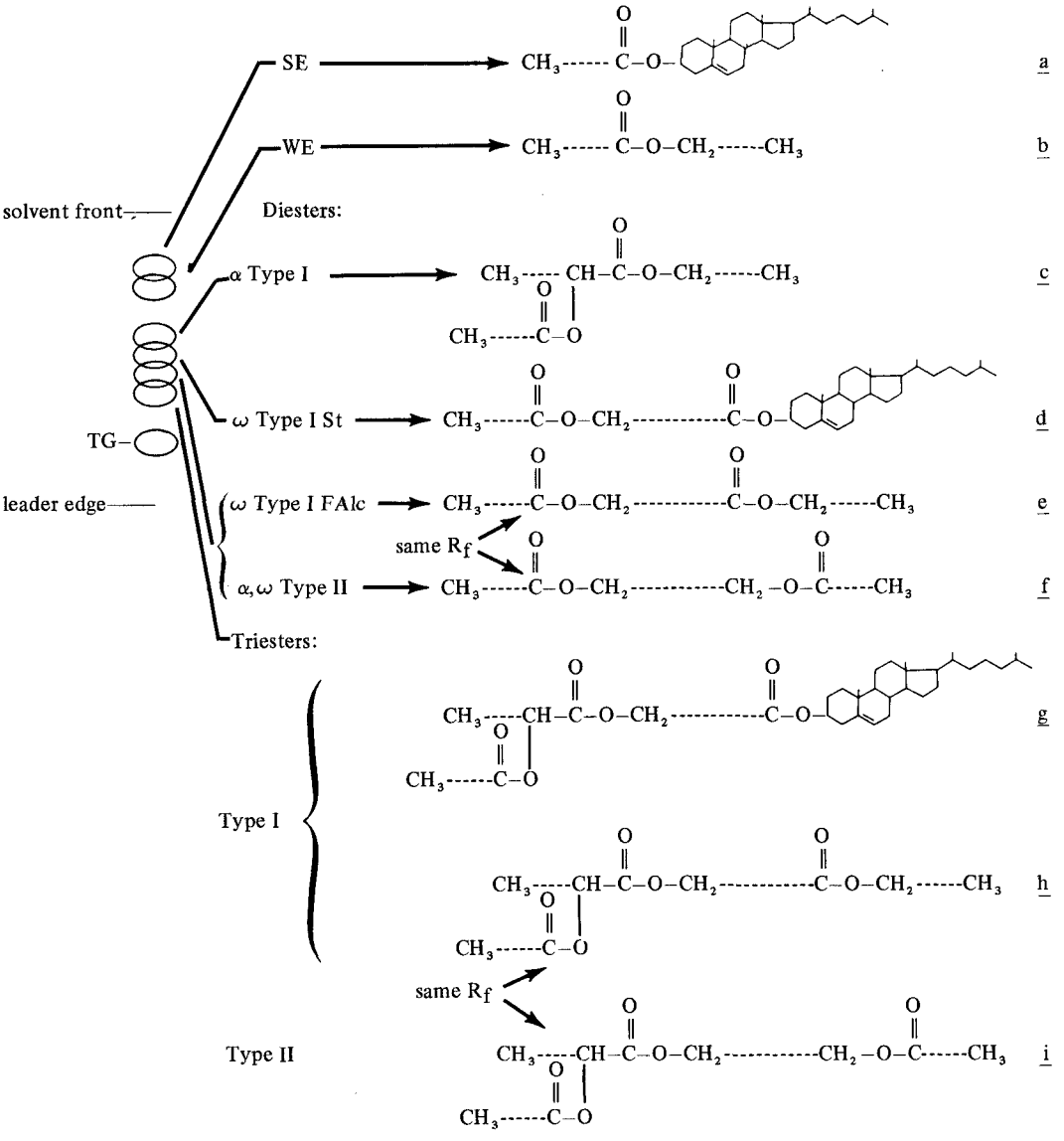

FIG. 3. Scheme showing the relative migration pattern of the mono, di and triesters on SiO_2. See text.

Fractions Nos. 10 to 14. Preparative TLC of these fractions on SiO_2 gave a diester band (Fig. 2) whose saponification products were FA, αOHFA and FAlc. GLC of the methyl esters of the fatty acids and the α-hydroxy fatty acids, and the acetates of the fatty alcohols, gave retention data matching those of standards. These data are listed in Table 2 and are discussed below.

To test for the possibility of another lipid class also being present in these diesters, the upper portion of the TLC band obtained from Fractions Nos. 11 and No. 12 was separated from the lower portion (Fig. 2) and individually saponified. Both portions gave similar results by TLC of the saponified products and GLC of the extracted fatty acids, α-hydroxy fatty acids and fatty alcohols, indicating homogeneity by this test. The weights recovered from the saponification products of these diesters were: α-hydroxy FAMEs, 170 μg, FAlcs, 201 μg and FAMEs, 187 μg. From the estimated average molecular weight of each component, a molar ratio of αOHFA:FAlc:FA of 1.0:0.93:1.1 was ob-

tained, which is approximately equimolar for all components. Within experimental error this supports the structure postulated.

Diesters ωType I and Possibly **FA-ωOHFA-St FA-ωOHFA-FAlc**

These diesters emerged from Column 211 in Fraction Nos. 12 to 22 (Table 1). If diesters FA-ωOHFA-FAlc occurred at all, they occurred in much smaller amounts than the amount of FA-ωOHFA-St. The latter diesters were separated from other material by preparative TLC on $Mg(OH)_2$ giving a broad band with an R_f of 0.1 when developed with 2.5% ethyl acetate in hexane. This adsorbent retains ring compounds with a flat surface more strongly than straight chain compounds of comparable molecular weight (23,24). Thus, molecules esterified to cholesterol or other similarly shaped sterols whose α side is relatively flat, are adsorbed more strongly on $Mg(OH)_2$ than those compounds esterified to straight chain fatty alcohols of comparable molecular weight. This chromatographic behavior of these two classes of esters on $Mg(OH)_2$ is quite the reverse of their behavior on SiO_2. TLC on SiO_2 of the material thus separated on $Mg(OH)_2$ as described above showed a single band, so the material was directly saponified without further purification. TLC of the saponified products by the 4-solvent linear development technique gave 3 bands: fatty acids, 163 μg, ω-hydroxy fatty acids, 307 μg (both as methyl esters), and sterols, 230 μg with respective R_f's of 0.36, 0.27 and 0.7. The sterols gave three peaks when they were subjected to capillary GC-MS and capillary GLC. The first to emerge was cholesterol, ~60%. The second was Δ7 cholestenol or 5α-cholest-7-en-3β-ol, ~35%. The third to emerge was present in too small an amount (~5% of the total sterols) to be identified. Authentic standards (Δ7 cholestenol from the Steroid Reference Collection, Medical Research Council, Westfield College, Hampstead, London) gave the same GLC retention times and nearly identical fragmentation patterns including a molecular ion at 386 by GC-MS. The molar ratio of FA:ωOHFA:St was 0.95:1.0:1.0, thus lending support, within experimental error, to the proposed structure. Compositions of the fatty acids of diesters FA-ωOHFA-St are listed in Table 2 and are discussed below.

Diesters α,ωType II **FA-α,ωdiol-FA**

These diesters were found in Fractions Nos. 13 to 23. They were separated from diesters FA-ωOHFA-St and other material also by the preparative TLC procedure on $Mg(OH)_2$ used above and were found in a broad band at R_f = 0.45. The saponification products of this band (which came from pooled Fraction Nos. 19 and 20) gave mainly fatty acids and α,ω-diols, but small amounts of α-hydroxy fatty acids, ω-hydroxy fatty acids and fatty alcohols also were present. The corresponding band at R_f = 0.45 from $Mg(OH)_2$ TLC of the earlier eluting pooled Fractions Nos. 15 and 16 also gave the same saponification products except that there were more α-hydroxy fatty acids, ω-hydroxy fatty acids and fatty alcohols present. The weights of these saponification products obtained from pooled Fractions Nos. 15 and 16 gave the following number of μmoles: FAlcs 0.12, α,ω-diols 0.37, FA 0.84, ωOHFA 0.24,

TABLE 2

Fatty Acids (as Methyl Esters) from Different Diester Fractions of Steer Meibomian Lipids (Column 211)

	Diesters αType I			
	Frac. 11			
	3 major homologs %			%
Short chain cluster (C12-C20)				
Saturates				
Normal	C16:0, 9.7	C14:0, 4.1	C18:0 2.0	16.3
Iso-branched	iC18:0, 7.2	iC14:0, 2.8	iC16:0 1.1	11.6
Anteiso-branched	aiC15:0, 18.8	aiC19:0, 5.9	aiC17:0 4.5	33.0
Monoenes				
Normal	C18:1, 10.1	C16:1, 3.3	C15:1 1.6	16.0
			Subtotal	76.9
Long chain cluster (C21-C31)				
Saturates				
Normal	C24:0, 0.5	C22:0, 0.4	C29:0 0.4	1.9
Iso-branched	iC26:0, 1.9	iC30:0, 1.4	iC24:0 0.9	5.0
Anteiso-branched	aiC25:0, 4.4	aiC27:0, 3.9	aiC29:0 1.5	11.7
Monoenes				
Normal	C27:1, 2.2	C28:1, 1.0	C26:1 0.7	4.5
			Subtotal	23.1
			Grand total	100.0

(Continued)

TABLE 2 (continued)

	Diesters ωType I-St							
	Frac. 15-16				Frac. 19-20			
	3 major homologs %			%	3 major homologs %			%
Short chain cluster (c12-C20)								
Saturates								
Normal	C16:0, 6.7	C18:0, 1.3	C15:0 0.8	10.4	C16:0, 5.0	C18:0, 1.6	C14:0 0.5	7.8
Iso-branched	iC18:0, 3.3	iC16:0, 1.4	iC14:0 0.7	5.6	iC18:0, 6.5	iC16:0, 0.8	iC20.0 0.6	8.3
Anteiso-branched	aiC15:0, 10.6	aiC17:0, 10.4	aiC19:0 1.2	22.3	aiC17:0, 6.4	aiC15:0, 5.8	aiC19:0 1.3	13.6
Monoenes								
Normal	C18:1, 34.5	C16:1, 21.7	C17:1 1.7	59.1	C18:1, 34.5	C16:1, 25.0	C17:1 1.7	59.5
			Subtotal	97.4				89.2
Long chain cluster (C21-C31)								
Saturates								
Normal	C24:0, 0.1	–	–	0.1	C24:0, 0.2	C26:0, 0.1	–	0.3
Iso-branched	iC26:0, 0.3	iC22:0, 0.3	iC24:0 0.1	0.8	iC22:0, 1.1	iC26:0, 0.8	iC21:0 0.7	2.6
Anteiso-branched	aiC25:0, 0.6	aiC27:0, 0.6	aiC29:0 0.3	1.7	aiC25:0, 2.7	aiC27:0, 1.4	aiC21:0 1.2	6.3
Monoenes								
Normal	–	–	–	–	C22:1, 0.8	C21:1, 0.8	–	1.6
			Subtotal	2.6				10.8
			Grand total	100.0				100.0

(Continued)

and αOHFA 0.24. The main diester present here was 0.37 μmoles of α,ωType II. If we assume that the FAlcs are 0.02 μmoles too high or the FAs are 0.02 μmoles too low we can account for all products. For example, assuming the former we could have the following products:

α,ωType II	FA-α,ωdiol-FA		
μmoles:	.37	.37	.37

STRUCTURE 1

αType I	FA-αOHFA-FAlc		
μmoles:	.05	.05	.05

STRUCTURE 2

ωType I	FA-ωOHFA-FAlc		
μmoles	.05	.05	.05

STRUCTURE 3

	αOHFA-ωOHFA	
μmoles:	.19	.19

STRUCTURE 4

Admittedly, Structure 4 is somewhat speculative, but there is no reason why it could not be formed, since the approximately 30 C-atoms in the chains of the ω-hydroxy fatty acids between the OH groups and CO_2H groups make these groups essentially independent. Molar ratios of the component diesters of pooled

TABLE 2 (continued)

	Diesters α,ω Type II							
	Frac. 15-16				Frac. 23			
	3 major homologs %			%	3 major homologs %			%
Short chain cluster (C12-C20)								
Saturates								
Normal	C16:0, 8.2	C18:0, 2.8	C15:0 1.5	13.7	C16:0, 3.7	C18:0, 1.2	C14:0 0.6	6.2
Iso-branched	iC16:0, 1.9	iC18:0, 1.5	iC14:0 0.7	4.7	iC18:0, 6.1	iC14:0, 1.0	iC16:0 0.7	7.8
Anteiso-branched	aiC15:0, 14.8	aiC17:0, 14.8	aiC19:0 2.1	31.7	aiC15:0, 9.5	aiC17:0, 5.1	aiC14:0 0.7	15.3
Monoenes								
Normal	C18:1, 33.6	C16:1, 8.1	C20:1 0.7	42.6	C18:1, 35.0	C16:1, 30.8	C14:1 2.5	70.4
			Subtotal	92.7				99.7
Long chain cluster (C21-C31)								
Saturates								
Normal	C21:0, 1.1	C23:0, 0.2	C26:0 0.1	1.4	–	–	–	–
Iso-branched	iC26:0, 0.6	iC28:0, 0.4	iC22:0 0.2	1.4	iC26:0, 0.1	iC24:0 0.1	–	0.2
Anteiso-branched	aiC27:0, 1.3	aiC25:0, 1.2	aiC29:0 0.5	3.2	aiC21:1 0.1	–	–	0.1
Monoenes								
Normal	C22:1, 0.8	C21:1, 0.5	–	1.3				
			Subtotal	7.3				0.3
			Grand total	100.0				100.0

Fractions Nos. 19 and 20 gave similar results. The sum of the weights of the diesters other than α,ω Type II are given in Table 1.

The purest preparation of α,ω Type II diesters that we were able to obtain was from Fraction No. 23, which gave a band at R_f = 0.51 on $Mg(OH)_2$ TLC. Saponified products from these diesters yielded 475 μg α,ω-diols and 201 μg fatty acids as methyl esters, giving a molar ratio of α,ω-diols to FA of 1.0:1.9 or approximately 1:2. Composition of the fatty acids of α,ω Type II diesters are given in Table 2 and are discussed below.

Triesters Type I

Triesters emerged from Colum 211 in Fractions Nos. 23 and 24. Preparative TLC of Fraction No. 23 on $Mg(OH)_2$ gave a broad band at R_f = 0.07 which, when further purified by preparative TLC on SiO_2, gave an intense upper band adjoined to a faint lower band. Saponification of the lipids from each band yielded the same products although in different amounts (Fig. 4). Corresponding substances from both lanes were pooled, all fatty acids methylated for GLC and weighed. Recovery of all substances gave the respective μmoles for FAlc, sterol, FA, ωOHFA and αOHFA as 0.05, 0.18, 0.24, 0.22 and 0.20. This gives a molar ratio of FA:αOHFA:ωOHFA:FAlc plus St of 1.2:1.0:1.1:1.1, or approximately 1:1:1:1. This molar ratio suggests the following two triester structues: FA-αOHFA-ωOHFA-St and FA-ωOHFA-αOHFA-FAlc, which would occur in the proportions 3.6:1, the molar ratio of sterols to wax alcohols. Interchanging the two hydroxy fatty acid moieties partially or totally may represent the actual structures, but the structures postulated would seem more plausible, because cleaving them at the middle ester linkage would

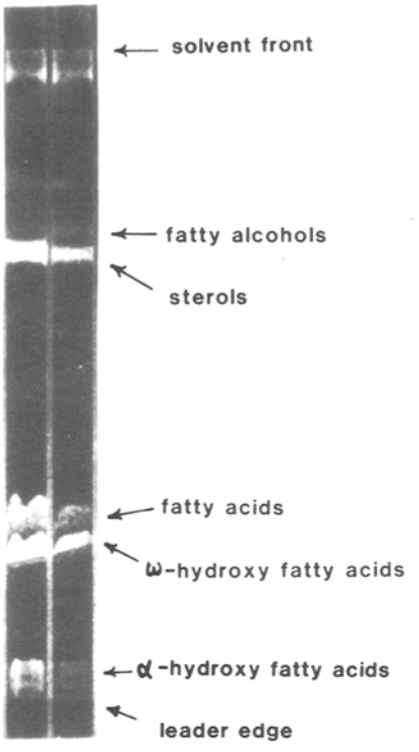

FIG. 4. TLC on SiO_2 of the saponified products of triesters Type I developed by the linear 4 solvent system described in the text. Fraction 23 Column 211 subjected to TLC on $Mg(OH)_2$; lipid from band with R_f = 0.07 subjected to TLC on SiO_2 yielding an intense band at R_f = .44 adjoining to a faint band at R_f = .41. Saponified products from intense band (250 μg) spotted in left lane and those from faint band (125 μg) spotted in right lane.

yield moieties already occurring in αType I and ωType I diesters. Analogously, one may argue that triesters with the structures FA-ωOHFA-αOHFA-St and FA-αOHFA-ωOHFA-FAlc are much less probable because the moiety αOHFA-St was not found in diesters αType I, and ωOHFA-FAlc occurs minimally if at all in diesters ωType I. Thus, in summary, the two most likely structures for triesters Type I are FA-αOHFA-ωOHFA-St and FA-ωOHFA-αOHFA-FAlc.

Triesters Type II

Fraction No. 23 also yielded another band (R_f = 0.23) when subjected to preparative TLC on $Mg(OH)_2$. The lipid from this band also gave an intense upper band adjoined to a faint lower band when subjected to preparative TLC on SiO_2. When the lipid from the intense band was saponified and the saponified products put through our TLC system (Fig. 5, Lanes 1 and 2) we obtained the following μmoles: FAlcs 0.24, sterols 0.05, α,ω-diols 0.10, FA 0.47, ωOHFA 0.16 and αOHFA 0.31. The total number of μmoles of all components with carboxyl groups was 0.94, and the total with hydroxyl was approximately equal at 0.96. The presence of significant amounts of α,ω-diols suggests the presence of some triesters Type II but does not exclude the presence of triesters Type I and possibly even some diesters. The following are some possibilities:

Type II triesters	FA-αOHFA-α,ωdiol-FA			
μmoles	.05	.05	.05	.05
Type II triesters	FA-ωOHFA-α,ωdiol-FA			
μmoles	.05	.05	.05	.05
Type I triesters	FA-αOHFA-ωOHFA-St			
μmoles	.05	.05	.05	.05
Type I triesters	FA-ωOHFA-αOHFA-FAlc			
μmoles	.06	.06	.06	.06

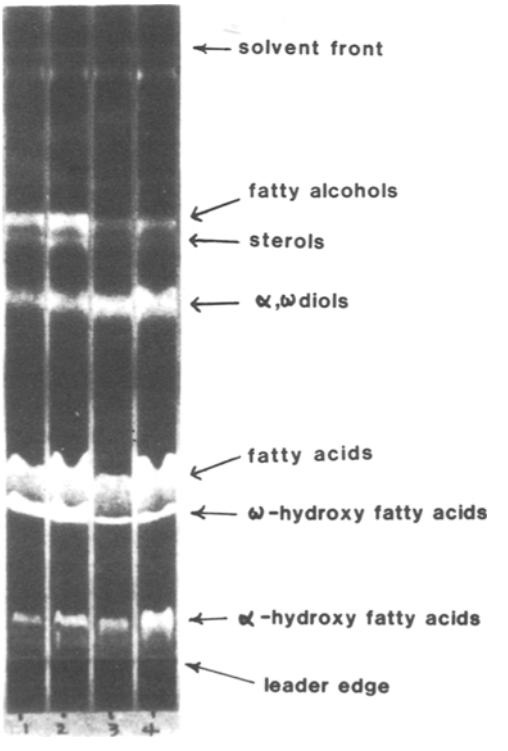

FIG. 5. TLC on SiO_2 of the saponified products of triesters Type II developed as in Fig. 4, obtained as described in text. Samples applied were 180 μg, 380 μg, 150 μg and 285 μg respectively for lanes 1 to 4. Structures postulated are tentative, see text.

This leaves 0.16 μmoles FA, 0.15 μmoles αOHFA plus 0.18 μmoles FAlc which is approximately correct for some α Type I diesters FA-αOHFA-FAlc, within experimental error. The difficulty is that αType I diesters should not elute this late in the Column 211. Perhaps it is a matter of selection of shorter chains for these compounds.

Fraction 24, when subjected to preparative TLC on $Mg(OH)_2$, gave a major band of R_f 0.23, the lipids of which were purified further by preparative TLC on SiO_2 to yield an intense upper band adjoined to a faint lower band as observed in Fraction No. 23. The saponified products of the intense band on SiO_2 of Fraction No. 24 (Fig. 5, Lanes 3 and 4) gave the following μmoles: FAlcs 0.09, sterols 0.02, α,ω-diols 0.18, FA 0.49, ωOHFA 0.11, and αOHFA 0.22. The total number of μmoles of carboxyl was 0.82 and hydroxyl was 0.80, suggesting the following possible substances:

Type I triesters	FA-αOHFA-ωOHFA-St			
μmoles	.02	.02	.02	.02
Type I triesters	FA-ωOHFA-αOHFA-FAlc			
μmoles	.09	.09	.09	.09
Type II triesters	FA-αOHFA-α,ωdiol-FA			
μmoles	.11	.11	.11	.11

This leaves 0.16 μmoles of FA and 0.07 μmoles of α,ωdiols which could be attributed to 0.07 μmoles of diesters FA-α,ωdiol-FA eluting with the triesters with an experimental error of 0.02 μmoles too many of FA.

The relative amounts of the different diesters present in the fractions of Column 211 were calculated from the weights recovered from TLC on SiO_2 and $Mg(OH)_2$, and are given in the totals of Table 1. Also given are estimates of the relatively small amounts of each diester present in Fraction Nos. 2 and 3 of Column 210, as judged by the intensity of TLC spots on SiO_2. Thus, steer meibomian lipids contain an estimated 1.3% αType I, 5% ωType I-St and 2.3% α,ωType II diesters. Table 1 also lists values obtained similarly for the triesters eluting with the more polar diesters. These comprise ~1% of steer meibomian lipids and do not include the triacylglycerols or other triesters that may migrate below them.

The fatty acids occurring in the three types of steer diesters isolated in this study are listed in Table 2. As mentioned earlier, when the different diesters emerge from the SiO_2 column, the fatty chains will change in that the more saturated chains will elute ahead of the more unsaturated chains, and the longer chains will elute ahead of the shorter chains. Perhaps a center cut or an average of an early and a late cut would give a fatty acid profile more representative of the total. Thus, for diesters ωType I-St the pooled Fractions Nos. 15 and 16 would be representative of the total. Considering these facts, there appear to be two fatty acid profiles: a one-cluster profile and a two-cluster profile. The first cluster is from C12 to C20, and the second from C21 to C31. The composition of the fatty acids of the αType I diesters is an example of the two-cluster profile; the fatty acids of the diesters ωType I and those of the α,ωType II belong largely to the one-cluster group. Anteiso chains are prominent in both profiles.

The wax alcohols of αType I diesters are quite similar to those of the wax monoesters (1), and the α-hydroxy fatty acids of αType I are similar to those of the total α-hydroxy fatty acids (2).

The very long chain difunctional group components, such as the ω-hydroxy fatty acids of ωType I diesters from pooled Fractions Nos. 15 and 16, Column 211, gave a profile similar to that of the total ω-hydroxy fatty acids reported earlier (2), and the α,ω-diols of α,ωType II diesters from pooled Fractions Nos. 19 and 20, Column 211, gave a profile similar to that of the α,ω-diols of the total unsaponifiables (4).

Human Meibum Polyesters

Human and steer meibum lipids when chromatographed alongside each other on the same TLC plate gave six bands in the diester and triester regions labeled DE-1 through DE-6 (Fig. 6). Each of these bands were scraped off from three such plates, pooled, extracted and the lipid obtained saponified. The saponified products were then chromatographed (Fig. 7) and the spots worked up and subjected to GLC as described above.

From our earlier data on the steer, we would expect that its DE-1, DE-2 and DE-3 bands would contain diesters αType I, ωType I-St and α,ωType II, respectively. In Figure 6, note that human DE-1 migrated farther than steer DE-1 but the migration of human DE-2 and DE-3 matched closely the respective migrations of DE-2 and DE-3 of the steer, although present in differing amounts.

If human DE-1 were αType I diesters as in the steer, we should have obtained α-hydroxy fatty acids. GLC analysis of the ultra small sample obtained from the human DE-1 band gave inconclusive evidence of this. This fact, coupled with the difference in migration be-

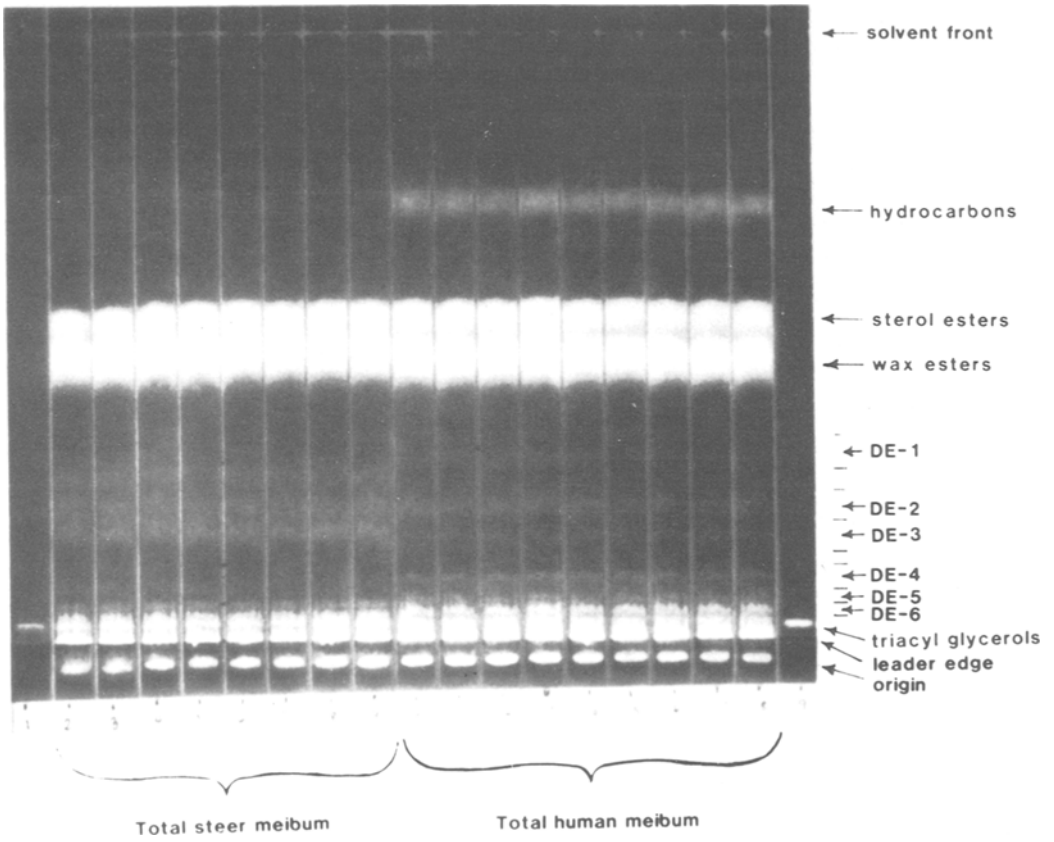

FIG. 6. TLC on SiO_2 of total steer and human meibum lipids. Plate was developed in hexane/benzene 55:45, v/v. Type of plate and spray used as in Fig. 1. Lanes 1 and 19 were 30 μg each of cholesterol and triolein standards. Lanes 2-9 and 10-18 are 300 μg each of total steer and human meibum lipids respectively.

tween the human and steer DE-1, makes it still an open question as to whether any αType I diesters occur in human meibum.

The saponified products of human DE-2 matched rather closely those of the steer DE-2. The amounts of sterol, FA and ωOHFA recovered (Fig. 7) and their GLC patterns are consistent with the expected structure for diesters ωType I-St. Capillary GLC of the sterols showed a chromatogram very similar to that of the steer except that there was only ~10% Δ7 cholestenol as determined by GC-MS. Similarly, human DE-3 showed amounts of α,ω-diols and fatty acids consistent with the structure of α,ωType II diesters. However, there was some contamination of DE-3 with DE-2 and vice versa.

The saponified products from DE-4, DE-5 and DE-6 from both the steer and the human showed enough similarity to lead one to believe that the triesters of both species also bear close resemblances to each other. Human meibum lipids also appear to contain small amounts of still a fourth difunctional group lipid component, namely, α,β-diols (see DE-4 and DE-6 of Fig. 7), and very faint traces of these diols also seem to be present in steer meibum lipids.

CONCLUSION

Approximately 90% of the lipids of steer and human meibum are esters of some 8 or 9 types, a large part of the remainder being free alcohols, sterols, acids and hydrocarbons. Each ester class in turn consists of a very large number of molecular species. It has been shown that the esterification of fatty acids with fatty alcohols for the human wax esters was a random process (25). Thus, 69 fatty acids of all fatty chain types times 40 fatty alcohols of all chain types form 2760 different molecular species of wax ester. If we add a third component such as the family of 11 α-hydroxy fatty acids, as found in diesters αType I, we get over

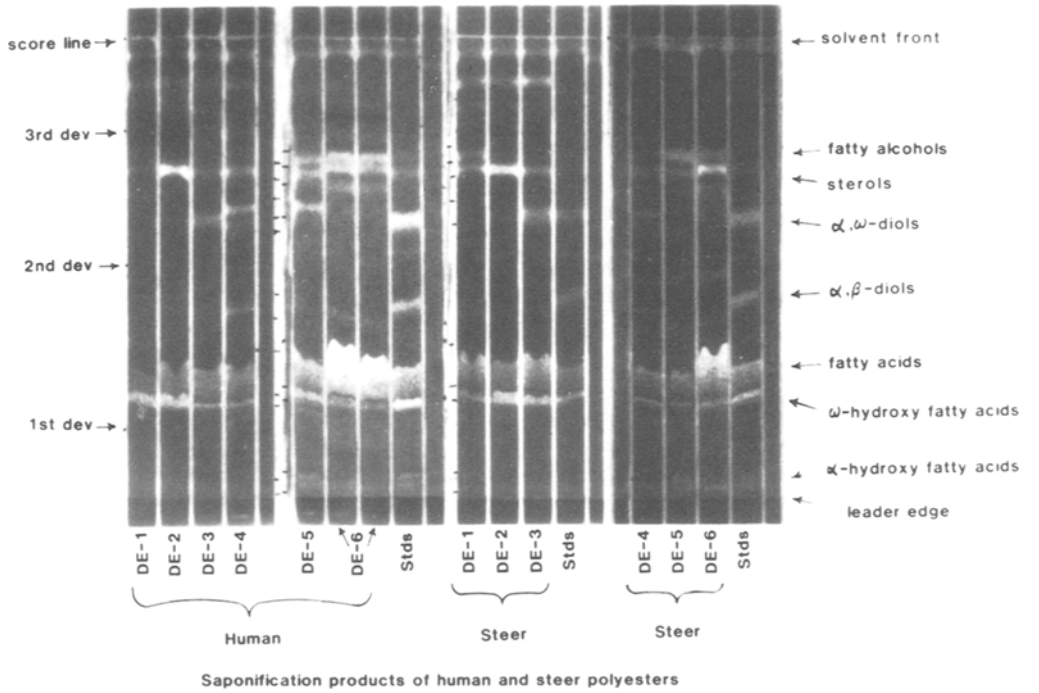

FIG. 7. TLC on SiO_2 of saponified products of bands in the diester region of total steer and human meibum lipids. Plates were developed as in Fig. 4. Type of plate and spray used as in Fig. 1. Samples applied on each lane from left to right were: Human meibum, DE-1 85 μg, DE-2 160 μg, DE-3 100 μg, DE-4 185 μg, DE-5 150 μg, DE-6 200 μg and 160 μg each; Steer meibum, DE-1 145 μg, DE-2 150 μg, DE-3 200 μg, DE-4 95 μg, DE-5 130 μg, DE-6 270 μg. Stds., 100-150 μg each, contained fatty alcohols, sterols, α,ω-diols, α,β-diols, fatty acids, ω-hydroxy fatty acids and α-hydroxy fatty acids.

30,000 molecular species, and a fourth component as in the triesters would bring the total number of molecular species to many hundreds of thousands. It would appear that no one of these molecules is more important than any of the others, but that all of them synergistically endow the mixture with those properties that enable the meibum to perform all of its functions.

One important physical property that this semi-solid lipid mixture must have is the correct viscosity (or fluidity) to enable it to flow out of the gland orifice onto the edge of the eyelid at the temperature of the eyelid. A second essential physical property is the right spreading characteristics over the wet tear surface so that it can keep the preocular tear film covered at all times by expanding and contracting with every blink and thus retarding evaporation of tear moisture. A third essential physical property is the correct adhesion to the outer skin of the eyelids so that a hydrophobic wall forms, thus preventing the skin from getting wet and swelling. Another function of this semi-solid wall is to direct the tears to flow into the puncta at the side of the nose and prevent them from overflowing onto the cheek. Finally, a fourth physical property required of this semi-solid lipid is that it be cohesive or sticky enough that the lipids of the upper and lower lids form a water tight seal when the eyes are closed during sleep, thus preventing the corneal surface from drying out. Whether meibum lipids have any useful chemical properties, such as toxicity to some pathogenic microorganism, remains yet to be determined.

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Biosynthesis of Lipids by Bovine Meibomian Glands

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ABSTRACT

Isolated bovine meibomian glands incorporated exogenous [1-^{14}C]acetate into lipids. Thin layer chromatographic analysis of the lipids showed that wax esters and sterol esters contained 61% of the total label. Radio gas liquid chromatographic analysis of the acid and alcohol moieties of both ester fractions showed the label was distributed equally between the two portions of the ester in both cases. Cholesterol and 5-α-cholest-7-en-3β-ol were the major labeled sterols, and anteiso-C25, anteiso-C27 and anteiso-C23 were the most highly labeled alcohols. The major labeled fatty acids in the wax esters were anteiso-C15, *n*-C16, anteiso-C17 and *n*-C18:1, whereas anteiso-C25 and anteiso-C27 were the major labeled acids in the sterol esters. The diester region with 6% of the total label contained labeled fatty acids and fatty alcohols each with anteiso-C25 as the major component and ω-hydroxy acids in which *n*-C32:1 was the major labeled component. The triglyceride fraction which contained 8% of the total lipids was composed of labeled fatty acids similar to those found in both sterol and wax ester fractions. Chromatographic analyses of the labeled lipids derived from exogenous labeled isoleucine showed that anteiso-branched products were preferentially labeled. The labeled triglyceride fraction derived from [U-^{14}C]isoleucine also contained esterified C15, C13, C11, C9, C7 and possibly shorter anteiso-branched acids.
Lipids 20:468-474, 1985.

INTRODUCTION

Sebaceous glands generate unique lipids for specialized functions (1,2,3). Meibomian glands located on the eyelids along the line of the eyelash are sebaceous glands which produce major lipids to retard evaporation of moisture from the tear film and to perform other functions important to the eye (4,5). Recent studies elucidated the nature of the major lipids produced by the bovine and human meibomian glands (6-8). The most dominant components of the bovine meibomian lipids are wax esters and sterol esters, each constituting about one-third of the total. Other components include diesters, triacylglycerols, free sterols, free fatty acids and polar lipids (8). The precise nature and composition of these relatively minor components have not been determined. A notable feature of the bovine meibomian lipids is the presence of a high proportion of anteiso-branched chains in both acids and alcohols. The presence of very long carbon chains is also noteworthy, and chains as long as C36 have been found in the ω-hydroxy acid fraction. Although speculative pathways can be suggested for the biosynthesis of the rather unusual mixture of lipids generated by the meibomian glands, no biosynthetic work has been reported heretofore. In this paper we report that exogenous labeled precursors are incorporated into the unique meibomian lipids by isolated glands.

MATERIALS AND METHODS

Materials

Intact eyelids from freshly killed steers (from Colfax Meat Packing, Colfax, Washington) were kept on ice for up to 4 hr before use. Na[1-^{14}C] Acetate (59 mCi/mmol) and L-[U-^{14}C]isoleucine (270 mCi/mmol) were purchased from New England Nuclear and ICN Chemical and Radioisotope Division, respectively. Silica gel 60G was obtained from EM Reagents, magnesium hydroxide (reagent grade) was from MCB and Celite 545-AW was from Johns-Manville.

Experimental

The eyelids were split with a razor blade in such a way that the row of glands was exposed. These glands were excised from the adhering connective tissue to give a preparation containing intact glands and slices of glands. This gland preparation was mixed with 50 μCi of the labeled substrate dissolved in 0.2 ml water to coat the tissue with labeled material, and the mixture was incubated for 5 hr at 30 C in a 5 ml beaker covered with tin foil. At the end of the incubation period, the tissue was transferred to a Ten-Broeck glass homogenizer and ground in 10 ml of a 2:1 mixture of chloroform and methanol. The homogenate was acidified with 6 N HCl and extracted by the method of Folch

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et al. (9). The $CHCl_3$ extract was washed with acidified water and evaporated to dryness under reduced pressure.

Fractionation of Meibomian Lipids

The total lipid recovered from the biosynthetic experiments was subjected to TLC on 1 mm layers of Silica Gel G with hexane/ethyl ether/formic acid (44:6:1, v/v/v) as the developing solvent. The lipid bands were visualized by viewing the plates under UV light after spraying them with 0.1% alcoholic solution of 2′,7′-dichlorofluoresceine. The thin layer chromatograms also were monitored for radioactivity with a Berthold scanner. Matching of the lipid bands with the radioactivity monitor response was used to identify the labeled fractions, and the lipids from silica gel obtained from the designated regions of the chromatogram were recovered by elution with a 2:1 mixture of chloroform and methanol. The ester fraction containing both sterol and wax esters was rechromatographed on magnesium hydroxide thin-layer plates as described (10). The fraction containing fatty acids and fatty alcohols, covering a relatively broad area, was rechromatographed on Silica Gel G with hexane/ethyl ether/formic acid (65:35:2, v/v/v) as the solvent. The acid and alcohol fractions were recovered separately and examined. The free sterol fraction also was rechromatographed with the above solvent system, and the free sterol was recovered and analyzed. An aliquot of the polar lipid fraction obtained from the origin in the first chromatography step was subjected to TLC on silica gel G with chloroform/methanol/glacial acetic acid/water (85:15:10:4, v/v/v/v) as the solvent with phosphatidylcholine and phosphatidylethanolamine as reference standards. The major part of the polar lipid fraction was transesterified with 14% BF_3 in methanol, and the products were subjected to TLC with hexane/ethyl ether/formic acid (65:35:2, v/v/v) as the solvent. The methyl ester and the alcohol fractions were recovered after monitoring the plates for ^{14}C and subjected to radio GLC (alcohols as acetates).

Preparation of Derivatives

Transesterification with methanol was performed by refluxing the lipid material dissolved in 0.5 ml toluene with 5 ml 14% BF_3 in methanol for 2 hr. For transesterification with butanol, a similar procedure was used with 14% BF_3 in *n*-butanol. Hydroxy-containing components were acetylated with a 2:1 mixture of acetic anhydride and pyridine at room temperature overnight.

Chromatography

TLC was performed on 20 × 20 cm, 0.5 or 1 mm thick Silica Gel G plates activated at 110 C overnight using the indicated solvent systems in lined tanks. TLC was done with 1 mm thick layers of $Mg(OH)_2$ activated for 2 hr at 110 C. Radio GLC of the non-steroidal compounds was done with a Perkin-Elmer Model 801 gas chromatograph attached to a Barber-Colman radioactivity monitor. A coiled stainless steel column (0.3 mm I.D. × 198 cm) packed with 5% OV-1 on 80-100 mesh Gas Chrom Q was used with Ar carrier gas at 80 ml/min. For methyl esters a temperature program from 180 C to 310 C (5°/min) was used; for acetylated alcohols and ω-acetoxymethyl esters a program from 220 C to 310 C (5°/min) was used. For radio GLC of sterol acetates a Gow-Mac gas chromatograph attached to a Nuclear-Chicago Model 735b radioactivity monitor was used with a coiled glass column (2.5 mm I.D. × 350 cm) packed with 5% OV-17 on 80-100 mesh Gas Chrom Q. The carrier gas (He) was at 58 ml/min, and the column temperature was 300. In all cases identification of the labeled components was made by co-chromatography with authentic compounds purchased from Analabs, West Haven, Connecticut, or with the meibomian components identified by combined GLC-MS. Structural analysis of the steroid acetates was done with a Hewlett-Packard 5840A gas chromatograph fitted with a 12 m capillary column of methylsilicon (ID 0.2-0.21) attached to a HP 5985 mass spectrometer.

Determination of Radioactivity

Liquid scintillation spectrometry was done with a Packard Tri-Carb liquid scintillation counter, Model 3255, using Omnifluor scintillation cocktail (New England Nuclear) with a counting efficiency of 72% as determined with ^{14}C-toluene standard. Inclusion of silica gel in the samples did not interfere with counting efficiency. The radio GLC conditions used gave detectable peaks for components containing >200 dpm.

RESULTS AND DISCUSSION

Incorporation of Labeled Acetate into Lipid Classes

The isolated gland preparation incorporated about 15% of the exogenous labeled acetate into lipids in 5 hr, demonstrating that glands and/or slices retained their lipid biosynthetic activity. To avoid selective labeling of only the most rapidly labeled components, the relatively long incubation time was chosen. TLC showed that the label in the lipids was distributed

among all of the major classes of meibomian lipids. The most heavily labeled fraction, containing 61% of the total label, was the ester fraction containing both wax and sterol esters. The lipid fraction recovered from a relatively broad region just below this ester region, designated as the diester fraction, contained about 6% of the label recovered in lipids. The triglyceride fraction contained about 8% of the label, whereas only 4.5% of the label was found in the slightly lower region containing free fatty acids and fatty alcohols. Free sterols and the polar material remaining at the origin contained 12% and 6%, respectively, of the label found in the lipids. Analysis of transesterification products of total lipids showed that 40% of the label was in the fatty acid portion, about 30% in sterols, 15% in primary alcohols and 5% in ω-hydroxy fatty acids.

Resolution of wax esters from sterol esters showed that the two types of esters contained 21% and 40%, respectively, of the label contained in the total lipids. In both cases the acyl portion and the alcoholic portion contained equal amounts of ^{14}C.

Distribution of ^{14}C in Sterols

Radio GLC of the sterol fraction as acetate showed that all of the ^{14}C was contained in two components. The major one, which contained 60% of the label, corresponded to cholesterol acetate, and the other, containing 40% of the label, had a slightly longer retention time. Since the identity of the sterols of the meibomian gland had not been reported, the sterol fraction obtained from the sterol esters of the meibomian lipids was subjected to combined GLC-MS. The major component was identified as cholesterol acetate. The component which had a slightly higher retention time gave a mass spectrum which was computer matched to that of 5-α-cholest-7-en-3β-ol acetate (Fig. 1). This component co-chromatographed with the second major labeled component of the sterol fraction. Thus, the major sterols, identified as cholesterol and 5-α-cholest-7-en-3β-ol, were the two major components synthesized from exogenous labeled acetate. The latter has been found as a major component in rat epidermis (11).

Distribution of ^{14}C in Fatty Alcohols

Radio GLC of the alcohols obtained from the wax esters showed that the major labeled fatty alcohols were anteiso-C25, anteiso-C27 and anteiso-C23; iso-C24 and iso-C26 also contained significant amounts of label (Table 1). *n*-Fatty alcohols were only minor components. This distribution of label was deduced from co-chromatography with endogenous meibomian components which were identified by GLC-MS, and these results reflected the chemical composition of the alcohols previously found in the wax esters (8).

Distribution of ^{14}C in the Acyl Portions of Wax and Sterol Esters

The distribution of ^{14}C among the fatty acids of the wax ester fraction was quite different from that found in the sterol ester fraction. The major labeled fatty acids found in wax esters were anteiso-C15, *n*-C16, anteiso-C17 and *n*-C18:1 (Fig. 2). On the other hand, the major labeled acids found in sterol esters were anteiso-C25 and anteiso-C27, with considerably less label in iso-C24, iso-C26, iso-C28, anteiso-C23 and anteiso-C21, and with detectable label in shorter homologs. Whether the observed clear differences in the distribution of label arise from compartmentation of synthesis or enzyme specificity is not known. In any case, the distribution of label is reflected in the chemical composition of the two classes of esters found in the present meibomian lipid samples (data not shown), and this composition was similar to that previously reported (8).

Distribution of ^{14}C in Components in the Diester Fraction

The diester region probably contained a mixture of lipids which individually constituted only minor components as observed in the thin layer chromatograms, and the total label contained in this fraction was also small (6%). Therefore, instead of attempting to isolate individual lipids, the mixture was transesterified with methanol and the products were subjected to TLC. The three components found were fatty acid methyl esters, fatty alcohols and ω-hydroxy acid methyl esters, containing 2%, 1% and 2%, respectively, of the label found in total lipids. Radio GLC showed that the major labeled fatty acid was anteiso-C25 (data not shown) as found in fractions designated as sterol esters, triglycerides and the free fatty acids. The distribution of label among the alcohols was quite similar to that observed in the alcohols of the wax ester fraction with anteiso-C25 as the major labeled component (data not shown). The labeled acetoxy methyl esters co-chromatographed with the endogenous compounds (Fig. 3), which were identified by the mass spectra of their trimethylsiloxy methyl esters which showed major diagnostic ions at M^+, M^+-15, M^+-31, M^+-47, M^+-90 and M^+-122. Thus the labeled ω-hydroxy acids were identified as C28:1, C30:0, C32:0, C32:1, C33:0 and C34:1. The most dominant component was C32:1, and it contained the

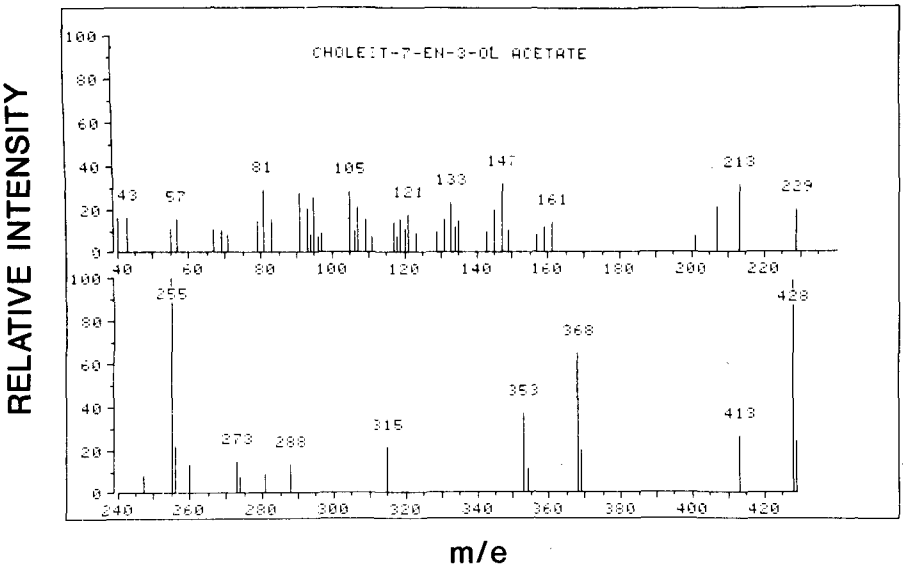

FIG. 1. Mass spectrum of 5-α-cholest-7-en-3β-ol from the sterol ester fraction of bovine meibomian gland.

TABLE 1

Distribution of ^{14}C in the Alcohol Components from the Wax Ester and Triglyceride Fraction Derived from [1-^{14}C] Acetate

Component	Composition (% of total)	
	Wax ester	Triglyceride
iso-C20	2.2	–
anteiso-C21	6.2	8.7
iso-C22	6.2	–
anteiso-C23	10.6	14.4
iso-C24	12.5	8.9
anteiso-C25	30.1	34.6
iso-C26	14.9	13.5
anteiso-C27	17.3	19.9

largest amount of label. This composition was similar to that reported previously (12). Since the structures of the components contained in the fraction designated diesters have not been established, only tentative conclusions can be drawn about the labeled material. Probably the fraction contains diesters composed of ω-hydroxy acids esterified to a fatty acid and fatty alcohol, and some of the ω-hydroxy acid also could be present as macrocyclic lactones similar to those reported to be present in the surface lipids of some mammals (13).

Distribution of Label in the Components of the Triglyceride Fraction

The fraction designated triglycerides probably contained other classes of lipids as pointed out previously (8). Thus, upon transesterification with methanol and analysis of the products by TLC, the label was found in primary alcohols (1% of that in the total lipids) and ω-hydroxy acid methyl esters (1%), although the major portion (5%) of the label was found in fatty acid methyl esters. The major labeled fatty acids were *n*-monounsaturated C16 and C18, anteiso-C25, anteiso-C27 and anteiso-C15. The ^{14}C-distribution in the primary alcohols (Table 1) and ω-hydroxy acids resembled those found in the alcohol portion of wax esters and the ω-hydroxy acids of the diester fraction, respectively.

Distribution of ^{14}C in Free Acids, Alcohols and Sterols

The small amount of label recovered in the thin layer chromatographic region below the triglycerides was contained mainly in free fatty acids which were similar to those found in the triglyceride fraction and fatty alcohols similar to those found in wax esters. All of the ^{14}C in the free sterol fraction was found in one component which co-chromatographed with the endogenous chemical which was identified as cholesterol by its mass spectrum. The 5-α-cholest-7-en-3β-ol, found as a significant component of the sterol ester fraction, was not found in the free sterol fraction.

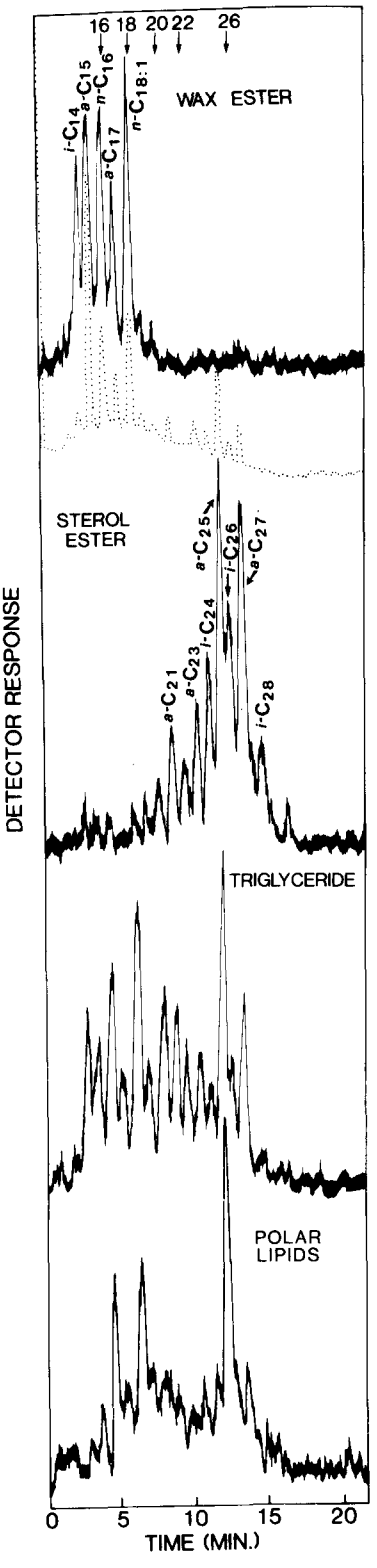

FIG. 2. Radio GLC of the methyl esters obtained from the wax ester, sterol ester, triglyceride and polar fractions derived from [1-^{14}C] acetate in bovine meibomian glands. Dotted line represents the flame ionization detector response due to coinjected unlabeled methyl esters obtained from the ester fraction of meibomian gland lipids; the peaks were identified by GLC-MS. Arrows indicate retention times of authentic *n*-methyl esters.

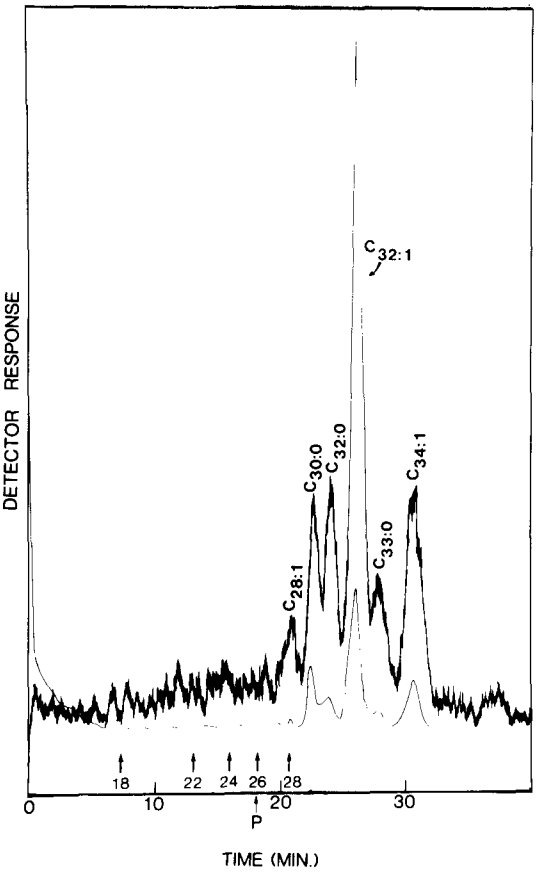

FIG. 3. Radio GLC of the ω-acetoxy methyl esters obtained from the diester (DE) fraction derived from [1-^{14}C] acetate in the bovine meibomian glands. Lower line represents the flame ionization detector response due to coinjected unlabeled ω-acetoxy methyl ester obtained from the meibomian gland lipids; the peaks were identified by GLC-MS. Arrows show retention times of authentic *n*-ω-acetoxy methyl esters. P indicates maximal temperature of the program.

Distribution of ^{14}C in the Polar Components

The polar lipids, recovered from the origin of the thin layer chromatogram, upon transesterification gave fatty acid methyl esters and a more polar fraction containing >50% and 20% of the radioactivity, respectively. Radio GLC of the polar fraction showed the presence of labeled ω-hydroxy acids similar to those indicated above and fatty alcohols up to C28 (data not shown). The major labeled acids were *n*-C16, *n*-C18 and anteiso-C25 (Fig. 2). The *n*-acids probably were constituents of the usual phospholipids and the anteiso-C25 acids might have been a constituent of a unique meibomian polar lipid component. In fact, TLC in a polar solvent system showed that phosphatidylcholine and phosphatidylethanolamine fraction contained 16% and 8%, respectively, of the radioactivity contained in the polar fraction.

Incorporation of ^{14}C into Lipids from L-[U-^{14}C] Isoleucine

Since isoleucine catabolism can provide anteiso-branched primers for the synthesis of anteiso-branched long chain compounds in other organisms (14-16), exogenous L-[U-^{14}C]isoleucine was used as a substrate. About 2% of the total radioactivity was incorporated into the crude lipids isolated after 5 hr of incubation with the labeled substrate. TLC of the total lipid showed that about 20% of the label was contained in wax and sterol ester fraction, 30% in a region with the polarity of triglycerides and 50% remained at the origin.

Resolution of the wax esters from sterol esters showed that the label from isoleucine was distributed equally between the two ester fractions. Transesterification and analysis of products by TLC showed that 54% and 79% of the label in the sterol ester and wax ester fractions, respectively, were in the fatty acid portions, the rest being in the alcoholic portions. Radio GLC of the fatty acids (as methyl esters) showed that the major labeled fatty acid in the wax ester was anteiso-C15, whereas in sterol esters the major acids were the anteiso homologs C25, C15, C27, C23 and C21 in the order of decreasing amounts of label (Fig. 4). Since all of the label incorporated into fatty acids from [U-^{14}C] isoleucine appears to be in the anteiso-branched fatty acids with odd numbers of carbon atoms, it is probable that the labeled anteiso-C5 primer derived from the degradation of isoleucine was incorporated directly into fatty acids.

When the triglyceride fraction was refluxed with 14% BF_3 in methanol only ∿10% of the label could be recovered as methyl esters. Because such a poor recovery could be due to

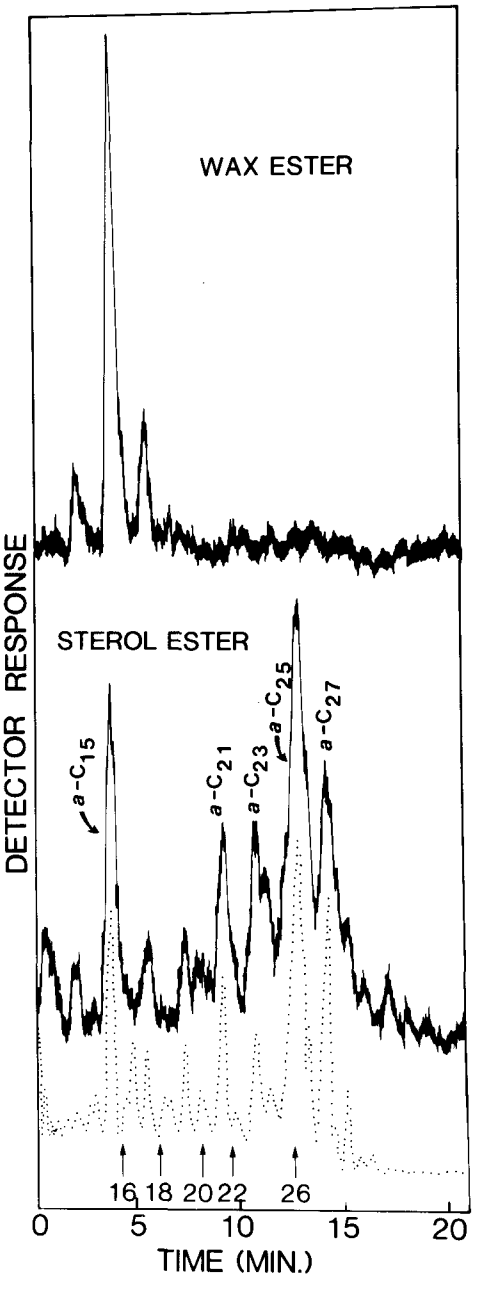

FIG. 4. Radio GLC of the methyl esters derived from the wax ester (top) and sterol ester (bottom) fractions derived from [U-^{14}C]isoleucine in bovine meibomian gland. Dotted line represents the flame ionization detector response due to the methyl esters present in the sample derived from the labeled sterol ester; the peaks were identified by GLC-MS on the same sample. The arrow indicates the retention time of authentic *n*-fatty acid methyl esters.

the presence of short-chain volatile acids, BF_3 in butanol was used for transesterification. The butyl esters recovered had nearly 20% of the original label contained in the fraction. Radio GLC of the butyl esters showed that the label was contained mainly in a homologous series of fatty acids which were identified tentatively by their retention times as C7, C9, C11, C13 and C15 anteiso-branched acids. Thus the triglyceride fraction contained the shortest acid so far identified in the meibomian lipids, and it appears probable that even shorter acids were present but were lost during isolation of the transesterification products because of the volatility. Such short chain components have not been detected in meibomian lipids previously, and the nature of lipids which contain such acids remains obscure.

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METHODS

Chromogenic Determination of Lipid Hydroperoxides by Sesamol Dimer

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ABSTRACT

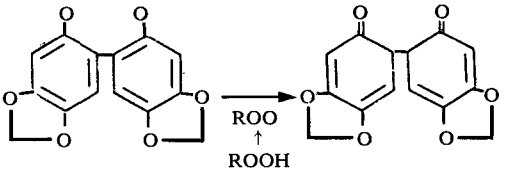
A specific chromogenic assay for lipid hydroperoxides was established. The principle of the assay was based on the reaction of sesamol dimer (I) and lipid hydroperoxides in the presence of hemoglobin (methemoglobin, oxyhemoglobin or carbonmonoxyhemoglobin) which produced violet-colored quinone or semiquinone dimer (II) having an absorption maximum at 550 nm (SD method). By this method, 0.1 μmol methyl linoleate hydroperoxide could be determined with higher sensitivity than the conventional peroxide value (POV) method. This method requires 0.2-5 mg of the sample for determination of lipid hydroperoxides. The extent of oxidation of oleic acid, linoleic acid, methyl oleate, methyl linoleate, soybean oil, sesame oil, hog fat, beef fat, chicken fat and the fat-fraction of processed foods could be determined, although the sensitivity varied with the samples and with the degree of autoxidation.
Lipids 20:475-481, 1985.

INTRODUCTION

Lipids can become rancid as a consequence of oxidation, which causes food deterioration. Lipid oxidation occurs by autoxidation of unsaturated fatty acid moieties such as oleate, linoleate and linolenate. The major primary products of autoxidation of the fatty acids are hydroperoxides, which in turn produce many secondary products, including aldehydes, ketones, acids, hydrocarbons and higher polymers. This sequence of reactions occurs not only in food deterioration but in biological systems in vitro and vivo (1).

The chemical determination of hydroperoxides (2) by Wheeler's method (3), the thiobarbituric acid (TBA) method (4), the ultraviolet measurement of conjugated diene (5) and the use of glutathione peroxidase (6) or horseradish peroxidase (7) have been used in the food and medical fields. Wheeler's method (peroxide value: POV) suffers from limited sensitivity, but spectrophotometric determination (8) or potentiometric titration of iodine (9,10) can increase the sensitivity. The TBA test is not spespecific to hydroperoxides but measures malonaldehyde and unsaturated aldehydes (2).

Previously we have demonstrated that sesamol dimer (I), which was prepared by partial oxidation of sesamol, can be converted readily into the violet-colored quinone or semiquinone dimer (II) with an absorption maximum at 550 nm by reaction with hydrogen peroxide and horseradish peroxidase (11). This reaction could be used for chromogenic assay of hydrogen peroxide and peroxidase (11,12). Preliminary experiments demonstrated that water-soluble linoleic acid hydroperoxide sodium salt effectively converted I into II in the presence of hemoglobin, whereas hydrogen peroxide and tert-butyl hydroperoxide had little activity under these conditions (13). It was suggested that the hydroperoxide-hemoglobin system produced some oxidative species which effectively oxidized I into II as shown in Scheme 1. This time, we evaluated this chromogenic assay as a specific method for determination of various water-insoluble hydroperoxides in autoxidized edible oils, fats and fat-containing foods.



I II

SCHEME 1

EXPERIMENTAL

Sesamol Dimer

Sesamol dimer (I) was prepared from sesamol by reaction with hydrogen peroxide and horseradish peroxidase according to the method described previously (11,12).

Hemoglobin Preparations

Human oxyhemoglobin (HbO_2) and methemoglobin (MetHb) were prepared according to the method previously described (14). Carbon-

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monoxy hemoglobin (COHb) was prepared by bubbling CO gas into HbO_2 solution. MetHb solution was treated with three equivalent amounts of KCN and passed through a Sephadex G-25 column to produce cyanomethemoglobin (CNMetHb). Commercial methemoglobin ($MetHb^c$) was obtained from Sigma Chemical Company (St. Louis, Missouri). Concentrations of hemoglobin (heme basis) in 0.1 M phosphate buffer (pH 7.0) were determined spectrophotometrically by use of molecular extinction coefficients: ϵ (577) = 14,600 for HbO_2 (15), ϵ (630) = 4,010 for MetHb and $MetHb^c$ (16), ϵ (569) = 13,400 for COHb (15), and ϵ (540) = 11,500 for CNMetHb (17). Horseradish peroxidase (Grade III, 105 units/mg) is a product of Toyobo Company, Ltd.

Lipid and Other Samples

Oleic acid, methyl oleate and methyl linoleate were the products of Tokyo Kasei Kogyo Company, Ltd. Linoleic acid was the product of Wako Pure Chemical Industries Ltd., Tokyo. Methyl linoleate hydroperoxide was prepared by autoxidation of methyl linoleate and successive column chromatography on silica gel, according to the method described previously (18,19). Peroxide value of the preparation was about 6,000 meq/kg, and the purity was more than 97% when compared to the theoretical value: 6,135 meq/kg. The preparation showed a single spot (Rf: 0.36) on a silica gel thin-layer chromatography (solvent: *n*-hexane-ethyl acetate, 4:1) and contained a negligible amount of methyl linoleate (Rf: 0.60) and the secondary degradation products when detected by iodine vapor, ultraviolet irradiation, spraying KI solution, and 2,4-dinitrophenylhydrazine solution. Commercial reagent-grade hydrogen peroxide (31%) was used, and the concentration was determined by iodometric titration.

Soybean oil was a product of Showa-sangyo Company, Ltd. Japan Pharmacopoeia sesame oil was used. Hog, beef and chicken fats were prepared from the corresponding fresh subcutaneous tissue by pressing and washing in hot distilled water. Fat-containing foods used in the experiments were instant Chinese noodle (Sapporo-Ichiban-Miso, Ramen, prepared by Sanyo-shokuhin Company, Ltd.), doughnut (prepared by Asahiseika Company, Ltd.) and fried rice-biscuit (Kabuki-age prepared by Amanoya Company, Ltd.).

Butylated hydroxytoluene (BHT) was obtained from Nikki-Universal Company, Ltd. dl-α-Tocopherol (α-Toc) was a product of Tokyo Kasei Kogyo Company, Ltd.

Autoxidation of Lipid Samples

Active oxygen method. Oils and fats were autoxidized by the active oxygen method (AOM) (20). A 20-ml portion of each sample was placed in a tube and aerated with purified air at the rate of 2.3 ml/sec and at 98 C. At regular intervals, a 0.5-1.0 g sample was removed to determine the extent of oxidation.

Ultraviolet irradiation. Fat-containing foods were irradiated by ultraviolet light. Each food was irradiated at room temperature and at a distance of 40 cm from two ultraviolet lamps (325 nm) (Toshiba Company, Ltd.). At regular intervals, a portion of the sample was removed to determine rancidity of the fat-fraction. Thus, about 50 g of each sample was extracted with 150 ml ethyl ether free from peroxides (Kanto Chemical Company, Inc.). The extract was washed with about 1/2 volume of distilled water after filtration through a filter paper, then was evaporated in vacuo after drying over anhydrous sodium sulfate. The residue was subjected to randicity tests.

Sesamol Dimer (SD) Method

A reaction mixture containing 0.60 ml of oxidized lipid sample solution in ethanol (or 2-propanol), 3.50 ml of 0.1 M phosphate buffer (pH 7.0), 0.50 ml of 3.0 mM sesamol dimer (I) in acetonitrile and 0.50 ml of hemoglobin solution in a tube protected from light was shaken vigorously for about 30 sec. It was required that hemoglobin solution be added at the final step, and the mixture was protected from light. Chloroform (5.0 ml) was added to extract the violet-colored quinone (II), and suspended water in the extract was removed by filtration through Toyo filter paper (Toyo Roshi Co. Ltd., Tokyo). Absorbance was measured at 550 nm by use of a Hitachi 100-10 spectrophotometer. One SD unit was defined as 0.1 of absorbance at 550 nm: this absorbance was the minimum required to estimate the amount of lipid hydroperoxides accurately.

POV Method

The amount of hydroperoxide was determined according to the method of Wheeler (3). One POV unit was defined as 1.0 ml of the titration volume of 0.01 N sodium thiosulfate: this volume is the minimum necessary to estimate lipid hydroperoxides accurately.

RESULTS

Reaction Conditions for Quinone (II) Formation from Sesamol Dimer (I) in a Methyl Linoleate Hydroperoxide-Hemoglobin System

Purified methyl linoleate hydroperoxide was

used as a model of lipid hydroperoxides to characterize the formation of violet-colored quinone (II) from sesamol dimer (I). The heterogeneous mixture of I, methyl linoleate hydroperoxide and hemoglobin in phosphate buffer (pH 7) was shaken, and the product (II) was extracted with chloroform. The amount of II produced was expressed in SD units based on the absorbance at 550 nm of the chloroform extract. It was required that I and water-insoluble methyl linoleate hydroperoxide be dissolved in acetonitrile and ethanol, respectively, before mixing, and that the hemoglobin solution be added at the final step so as not to be denatured before reaction with the hydroperoxide.

Formation of II in reaction of I with 0.5 μmol methyl linoleate hydroperoxide was estimated with several kinds of hemoglobin under the conditions described in the Experimental Section (Fig. 1). Hemoglobin preparations tested were methemoglobin (MetHb), commercial methemoglobin (MetHb^c), oxyhemoglobin (HbO_2), carbonmonoxy hemoglobin

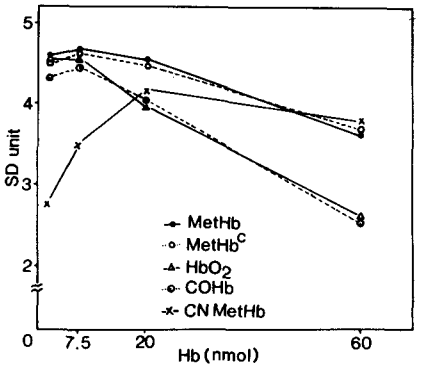

FIG. 1. Formation of II in reaction of I with methyl linoleate hydroperoxide in the presence of hemoglobin. I was treated with methyl linoleate hydroperoxide (0.5 μmol; 0.1 POV unit) in the presence of various amounts of several kinds of hemoglobin. One SD unit was defined as 0.1 of absorbance at 550 nm.

(COHb) and cyanomethemoglobin (CNMetHb). Each hemoglobin species exhibited an optimum amount for production of II. Optimum amounts of MetHb (or MetHb^c) were between 1.5-30 nmol. HbO_2 and COHb were as active as MetHb at amounts from 1.5-8 nmol, but the use of higher amounts produced II in lower yields. CNMetHb was much less active than the other hemoglobin species.

Sesamol dimer (I) was treated with various amounts of methyl linoleate hydroperoxide in the presence of 30 nmol MetHb. While the amount of MetHb was larger than that for the optimal conditions, it produced 90% of the maximum coloration. SD units increased with the amount of methyl linoleate hydroperoxide, and the relationship between the POV and SD units was linear (Fig. 2). One SD unit corresponded to 0.025 POV unit (0.125 μmol) of methyl linoleate hydroperoxide. The results indicate the SD method could determine 0.1 μmol of methyl linoleate hydroperoxide accurately.

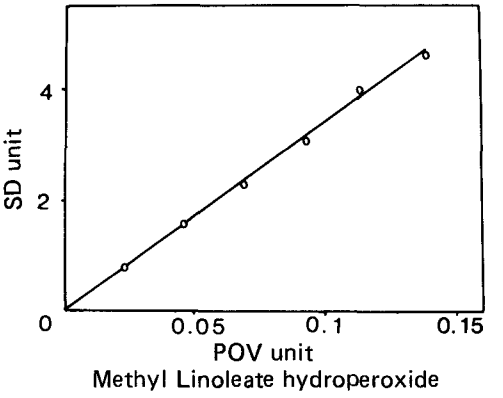

FIG. 2. Relationship between POV unit of methyl linoleate hydroperoxide and SD unit in reaction of I in the presence of 30 nmol MetHb. The amount of methyl linoleate hydroperoxide was expressed by the calculated POV unit values. One POV unit was defined as 1.0 ml of the titration volume of thiosulfate solution.

Linear relationships between POV and SD of methyl linoleate hydroperoxide were obtained with all hemoglobin species except CNMetHb. Almost equal SD values were obtained when 1.5-30 nmol MetHb (MetHb^c) and 1.5-8 nmol HbO_2 or COHb were used. Use of a larger amount of hemoglobin (more than 50 nmol of HbO_2) inhibited the formation of II, and the calibration curve was shifted toward the right. It is likely that a large amount of the protein consumed the active species produced by interaction of the hydroperoxide with hemoglobin, thus inhibiting the formation of II. The amount of I (1.5 μmol) was sufficient to determine the hydroperoxide, because 3 μmol of I did not increase the SD value further. There were some limitations to the SD method. For instance, the heterogeneous reaction mixtures should be shaken vigorously and extracted within 1 min to obtain reproducible results. Incubation of the reaction mixture at 37 C for 30 min decreased values by about 20%. Furthermore, the extracted

quinone (II) was susceptible to light. When the extract was protected from light, the SD value remained unchanged for 100 min. When it was exposed to sunlight, the violet-color greatly diminished within 10 min and the SD value decreased to less than 10% of the initial value (Fig. 3). While the reaction was little affected by a small amount of an antioxidant, butylated hydroxytoluene (BHT) or dl-α-tocopherol (α-TOC), a large amount of the antioxidant suppressed the formation of II (Table 1). More than an equivalent amount of the antioxidant was required to inhibit the reaction by 50%, except α-Toc which was somewhat more inhibitory.

In order to investigate the specificity of the reaction, formation of II by methyl linoleate hydroperoxide and by hydrogen peroxide were compared. Whereas methyl linoleate hydroperoxide produced II in a high ratio of 30-40 of SD value/POV, hydrogen peroxide produced II in a ratio of less than 1 (Fig. 4.). In contrast, hydrogen peroxide produced a much higher amount of II and methyl linoleate hydroperoxide produced a lesser amount of II when horseradish peroxidase was used instead of hemoglobin. Therefore, methyl linoleate hydroperoxide could be determined specifically in the presence of hemoglobin, while hydrogen peroxide could be determined specifically in the presence of horseradish peroxidase. The results were similar to the earlier findings demonstrating that linoleic acid hydroperoxide sodium salt showed much more sensitivity than hydrogen peroxide and tert-butyl hydroperoxide in the SD method (13).

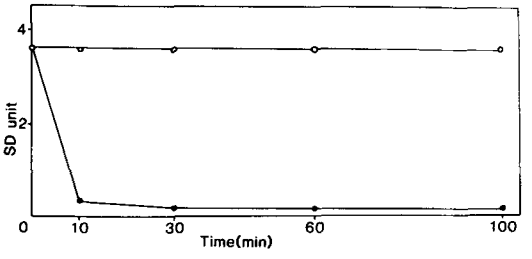

FIG. 3. Stability of II in chloroform extract. I was treated with methyl linoleate hydroperoxide (0.1 POV unit) in the presence of 30 nmol MetHb, and the chloroform extract was kept at room temperature in the dark (○) and exposed to sunlight (●).

Measurement of Hydroperoxides in the Autoxidized Unsaturated Fatty Acids and Their Methyl Esters

Oleic acid, linoleic acid, methyl oleate and methyl linoleate were autoxidized by the active

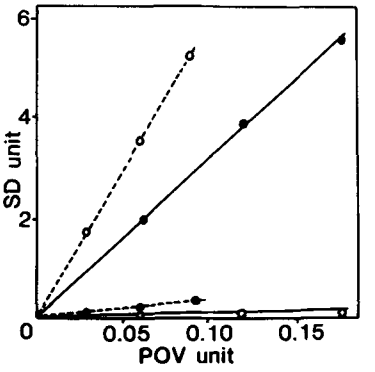

FIG. 4. Formation of II in reaction of I with methyl linoleate hydroperoxide and hydrogen peroxide. I was treated with methyl linoleate hydroperoxide (●) or hydrogen peroxide (○) in the presence of 7.5 nmol COHb (—) or 0.5 mnol horseradish peroxidase (- - - -).

TABLE 1

Effect of Antioxidants on the Formation of II in Reaction of I with Methyl Linoleate Hydroperoxide Preparations in the Presence of 30 nmol MetHb

Sample	Hydroperoxide μmol	Antioxidant μmol	SD unit	% Inhibition
Methyl linoleate hydroperoxide	0.25	–	1.75	–
	0.25	BHT, 2	1.05	40
	0.50	–	3.35	–
	0.50	BHT, 2	2.45	27
Autoxidized methyl linoleate (AOM, 8 hr)	0.60	–	2.90	–
	0.60	BHT, 4	1.20	59
	0.60	BHT, 8	0.85	71
	0.60	α-Toc, 0.2	2.00	31
	0.60	α-Toc, 0.4	1.25	57

A 0.20-ml solution of ethanol with or without the antioxidant was added to the reaction mixture.

oxygen method (AOM). Hydroperoxides formed in the autoxidized samples were measured by both SD and POV methods. The SD method required 0.2-5 mg of the sample dissolved in 0.6 ml ethanol, and the mixture was treated in the presence of 30 nmol MetHb under standard conditions. The POV method required 50-500 mg of the sample. Results are summarized in Figure 5. SD values linearly increased with the amount of every autoxidized sample (Fig. 5 inserts). Time courses of SD values against autoxidation time showed a parallel increase and decrease with those of POV. There were, however, significant differences in the ratio of the SD value/POV with the fatty acid samples and with the degree of autoxidation of the individual fatty acid sample: the ratio was 32 (7-hr-oxidized oleic acid), 42 (4-hr-oxidized linoleic acid), 16 (22-hr-oxidized methyl oleate), 23 (1-hr-oxidized methyl linoleate), 21 (8-hr-oxidized methyl linoleate) and 8 (47-hr-oxidized methyl linoleate). This may be due to the difference in ability of the fatty acid hydroperoxides to produce active species for oxidation of I or to the inhibition of the oxidation of I by a large number of secondary products in the autoxidized samples. Nevertheless, the method might be useful for determination of hydroperoxides.

Measurement of Hydroperoxides in the Autoxidized Oils and Fats

Soybean oil, sesame oil, hog fat, beef fat and chicken fat were autoxidized by the AOM. Two to five mg of each autoxidized sample were dissolved in 0.6 ml 2-propanol and subjected to the SD method. The SD values were proportional to the amount of the autoxidized sample (data not shown). Time courses of SD values against autoxidation time showed a parallel increase and decrease compared to POV (Fig. 6). The ratio of SD value/POV was 13 (24-hr-oxidized soybean oil), 13 (32-hr-oxidized sesame oil), 13 (22-hr-oxidized hog fat), 20 (96-hr-oxidized beef fat) and 11 (36-hr-oxidized chicken fat).

Processed foods such as instant Chinese noodle, fried rice-biscuit and doughnut, all containing fats, were irradiated by ultraviolet light for up to 14 days. The extent of oxidation of the fat fraction was monitored by the SD and POV methods after the fat fractions were extracted with ethyl ether (Fig. 7). In every case, SD and POV values were well correlated. The ratio of SD value/POV ranged from 5 to 15.

These results demonstrate that the SD method was sensitive for determination of lipid hydroperoxides, although there were significant differences in the ratio of SD value/POV with the samples and the degree of autoxidation.

DISCUSSION

It has been shown that sesamol dimer (I), prepared by partial oxidation of sesamol, was converted readily into violet-colored quinone or semiquinone dimer (II) by reaction with hydrogen peroxide and horseradish peroxidase (11),

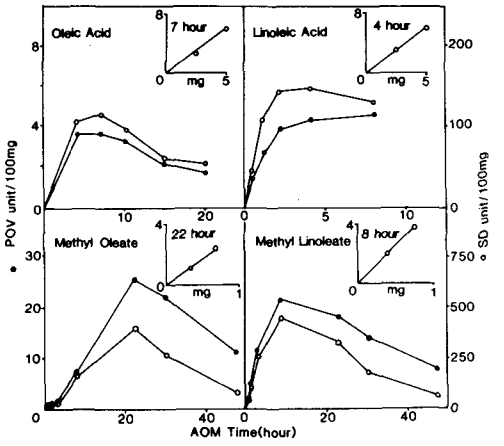

FIG. 5. Extent of autoxidation of fatty acids and their methyl esters estimated by SD and POV methods. Each fatty acid or the methyl ester was autoxidized in AOM. The hydroperoxides in the autoxidized sample (0.2-5 mg) in 0.6 ml ethanol were determined by the SD method (30 nmol MetHb). Inserts indicate representative calibration curves of the latter method.

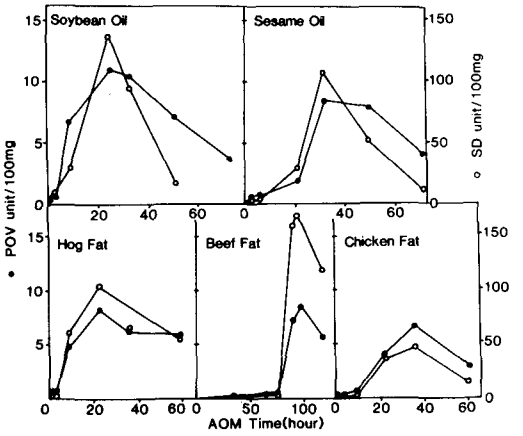

FIG. 6. Extent of autoxidation of vegetable oils and animal fats estimated by the SD and POV methods. Each oil or fat was autoxidized in AOM. The hydroperoxides in the autoxidized sample (5 mg) in 0.6 ml 2-propanol was determined by the SD method (30 nmol MetHb).

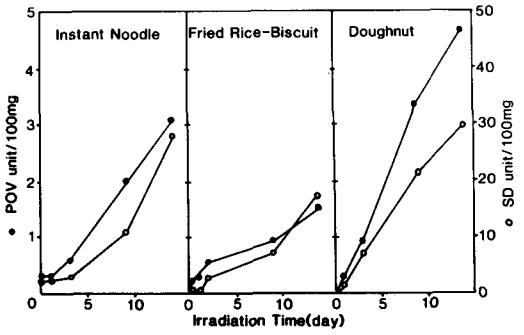

FIG. 7. Extent of autoxidation of the fat fraction of the processed foods estimated by the SD and POV methods. Fat-containing foods were irradiated by ultraviolet light. The fat fraction was extracted with ethyl ether, and the hydroperoxides in the fat (5 mg) in 0.6 ml 2-propanol were determined by the SD method (30 nmol MetHb).

and the reaction was useful for determination of hydrogen peroxide in foodstuffs (12). Other experiments showed that I was converted into II by reaction with water soluble linoleic acid hydroperoxide sodium salt and hemoglobin (13). The present experiment demonstrated that the system containing I and hemoglobin (SD method) was useful for determining various kinds of lipid hydroperoxides. This method could determine 0.1 μmol methyl linoleate hydroperoxide, and the relationship between the amount of the hydroperoxide and the formation of II was linear. Sensitivity of the SD method to methyl linoleate hydroperoxide was compared with that of the POV method. In the present experiments, 1 POV unit was defined as 5 μmol of the hydroperoxide which required 1.0 ml of 0.01 N thiosulfate solution. The ratio of SD values to POV was 30-40 (Fig. 2), which indicates the sensitivity of the SD method was 30-40 fold higher than the POV method (Wheeler's method). However, by using 0.002 N thiosulfate solution (21), it is possible to determine 1 μmol hydroperoxide. Thus, the sensitivity of 0.1 μmol hydroperoxide for the SD method is 6-8 fold higher than the alternative POV method.

The SD method could determine the extent of oxidation of oleic acid, linoleic acid, methyl oleate, methyl linoleate, soybean oil, sesame oil, hog fat, beef fat, chicken fat and the fat fraction of processed foods as well as the POV method, although significant differences in the ratio of SD values/POV values were observed with the samples and the degree of autoxidation. This method requires only 0.2-5 mg of the sample for determination of the hydroperoxides.

Hemoglobin produced reactive species for the oxidation of I by interaction with methyl linoleate hydroperoxide and lipid hydroperoxides, but it was much less active with hydrogen peroxide. Thus, the SD method was specific to lipid hydroperoxides, although the sensitivity was different with lipid samples of different degrees of autoxidation. In order to obtain reproducible results, several precautions must be taken for the SD method. The heterogeneous reaction mixtures containing I, lipid sample and hemoglobin should be shaken vigorously after the addition of hemoglobin, and the unstable violet-colored quinone (II) should be extracted rapidly in chloroform and protected from light before measurement of absorbance.

Several enzymatic assays of lipid hydroperoxides have been demonstrated, including the use of glutathione peroxidase (6) and horseradish peroxidase (7). Because glutathione peroxidase was active to both hydrogen peroxide and lipid hydroperoxides, it is necessary to destroy hydrogen peroxide by catalase prior to the addition of glutathione peroxidase for the selective determination of lipid hydroperoxides (6). Yamaguchi (7) used the system comprised of 4-aminoantipyrine, N,N-dimethylaniline and horseradish peroxidase for determination of methyl linolenate hydroperoxide, which produces the imine-quinone derivative having an absorption maximum at 558 nm. While the system was sensitive to methyl linolenate hydroperoxide, it also was sensitive to other peroxides such as hydrogen peroxide, methyl hydroperoxide and ethyl hydroperoxide. The high affinity of hemoglobin for lipid hydroperoxides made it possible to establish a specific chromogenic determination of lipid hydroperoxides in a food or nutritional field using the SD method.

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Determination of the Amounts of Free Arachidonic Acid in Resident and Activated Rabbit Alveolar Macrophages by Fluorometric High Performance Liquid Chromatography

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ABSTRACT

An improved method of high performance liquid chromatography (HPLC) has been developed for the separation and quantitation of low levels of free fatty acids as they occur in mammalian tissues. The fatty acid analysis is based on the esterification of the carboxylic group with 9-anthryldiazomethane (ADAM). HPLC separation and fluorescence measurement of fatty acid ADAM esters allow the determination of pmole amounts of fatty acids.

The amounts of free fatty acids of resident and activated alveolar macrophages were determined by the fluorometric HPLC method. There were approximately 2 $\mu g/10^6$ cells of free fatty acids. In resident macrophages, free 20:4 was a minor component (0.8% of total free fatty acids), while significant amounts of 20:4 were found in the total glycerophospholipids, representing 16.6% of the total fatty acids. A marked increase in amounts of 20:4 (8 times) occurred in activated macrophages stimulated for 1 hr with opsonized zymosan. Small but significant increases (1.5 times) also occurred in other fatty acids. These results show that the release reaction of fatty acids was not selective for 20:4 in alveolar macrophages after the challenge with opsonized zymosan.
Lipids 20:482-487, 1985.

INTRODUCTION

Arachidonic acid (20:4) plays an important role as a precursor for novel eicosanoids such as prostaglandins and leukotrienes. The synthesis of these eicosanoids is limited by the availability of free 20:4, which must be liberated from esterified stores such as glycerophospholipids or triacylglycerol (1). Therefore, the determination of the amounts of free 20:4 is important for elucidation of the mechanism that regulates the production of bioactive eicosanoids. Little work has been done to determine the free 20:4 in mammalian tissues, because the level of free 20:4 in cytoplasm is quite low compared with other fatty acids.

Although GLC is a common technique for quantitating fatty acids, it is not sensitive enough to detect the low level of free 20:4. Also, separated fatty acids could not be recovered from the column. Recently, reverse-phase HPLC has facilitated excellent separation of simple fatty acids or a methyl ester mixture (2-4), but the strength of absorbance (below 205 nm) of fatty acids depends on the number of double bonds in the fatty acids, and quantitation of each fatty acid requires the collection and gas liquid chromatographic analysis of all HPLC separated compounds. The low sensitivity and complexity of the quantitation of each fatty acid have led to the use of aromatic derivatives which have specific and strong absorbance, such as p-methoxyanilide (5), 2-naphthacyl- (6), phenacyl- (7), p-bromophenacyl- (8), methoxyphenacyl (9), 4-bromomethyl-7-methoxycoumarin (10) and 9-aminophenanthrene (11) esters, to enhance detection. Of many prelabelling reagents, 9-anthryldiazomethane (ADAM) (12, 13) has several advantages over other reagents. These include faster derivatization; no catalyst, either acid or base, is required; no heating; high reactivity with free fatty acids, and high fluorescence intensity.

In most studies of the separation and quantitation of fatty acid derivatives using the above reagents, standard mixtures of fatty acids were used. Fluorometric HPLC methods with ADAM derivatives have not been applied for the separation or quantitation of fatty acids of mammalian tissues.

In the present work, we used the ADAM derivatives of free fatty acids for the separation and quantitation of all major free fatty acids of resident alveolar macrophages and investigated the change in the amounts of free 20:4 in activated alveolar macrophages.

MATERIALS AND METHODS

Materials

Standard fatty acids were purchased from Nu-Chek-Prep (Elysian, Minnesota). Zymosan and cytocharasin B were from Sigma Chemical Co. (St. Louis, Missouri). 9-ADAM was obtained from Funakoshi Co. (Tokyo, Japan). HPLC

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grade organic solvents and distilled water were purchased from Wako Pure Chemical Ind. (Osaka, Japan).

Instruments

A Hitachi (Tokyo, Japan) Model 655 high performance liquid chromatograph equipped with a fluorescence detector (RF 530, Shimadzu Co., Kyoto, Japan) was used for the separation of fatty acid ADAM esters. The reverse-phase column, Zorbax ODS (4.6 mm x 25 cm, 5-6 μm particle size) was from DuPont Co. (Wilmington, Delaware). The column temperature was controlled with a block heater (Scientific System Inc., State College, Pennsylvania).

Derivatization of Free Fatty Acids and Analytical Procedures

Derivatization of free fatty acids was performed essentially as described in the original paper of Nimura and Kinoshita (12). A standard mixture solution or free fatty acids prepared from alveolar macrophages was dissolved in 100 μl methanol. ADAM (1 mg/ml) was dissolved in one drop of acetone and HPLC grade methanol. 100 μl of the ADAM solution was added to the sample solution. The reaction mixtures were allowed to stand for 4 hr at room temperature in the dark. After the reaction was complete, an aliquot (20 μl) of the solution was injected directly into the chromatograph.

Fatty acids ADAM esters were separated on the Zorbax ODS column at 30 C. The mobile phase was acetonitrile/isopropanol/water (90:9:1, v/v/v). The flow rate was 1.2 ml/min. Each peak was detected with the fluorescence detector (excitation at 365 nm, emission at 412 nm). The 17:0 ADAM ester was used as the internal standard for the quantitation of fatty acids. For the determination of free fatty acids by GLC, free fatty acids were methylated with diazomethane. The compositions of fatty acids of glycerophospholipids, triacylglycerol and diacylglycerol were determined by GLC analysis after transmethylation of the esterified fatty acids with sodium methoxide (14). Methyl esters of fatty acids were analyzed by GLC on a glass column packed with 15% diethyleneglycol succinate on Chromosorb W at 195 C.

Preparation of Alveolar Macrophages from Rabbits

Alveolar macrophages were harvested from rabbit lung according to the method of Myrvik et al. (15). Contaminating erythrocytes were removed by osmotic lysis, and the cells were washed 3 times with isotonic saline. Examination of Giesma stained smears showed that 95% of the lung washed cells were alveolar macrophages. From 3 to 8×10^7 cells were obtained from a single animal. The collected cells were suspended in 10 ml of 10 mM Hepes-Eagle's minimal essential medium (MEM) (pH 7.2). The effect of opsonized zymosan on the release of 20:4 was investigated by incubating alveolar macrophages with 10 mM Hepes-MEM medium containing cytocharasin B (2 μg/ml) in the presence or absence of opsonized zymosan (1 mg/ml). The reactions were stopped by adding organic solvent (chloroform/methanol, 1:2, v/v).

Extraction and Separation of Free Fatty Acids from Alveolar Macrophages

Cellular lipids were extracted by the method of Bligh and Dyer (16). Total lipids were fractionated into free fatty acids, triacylglycerol and glycerophospholipids by TLC with development with petroleum ether/diethyl ether/acetic acid (75:25:1, v/v/v). Each band was scraped off from the TLC plate and extracted by Bligh and Dyer's method. Free fatty acids were stored in chloroform at -20 C prior to derivatization.

RESULTS AND DISCUSSION

ADAM is the most favored reagent for the formation of carboxylic acids ester because of its high reactivity under mild conditions. More than 95% of fatty acids were converted to ADAM esters after 2 hr reaction (data not shown). The high reactivity of ADAM with free fatty acids was in good agreement with the results of the previous papers (10,13). A typical HPLC chromatogram of a synthetic mixture of fatty acid derivatives is shown in Figure 1. Binary mixtures of acetonitrile with methanol or water could not separate the mixture of 16:0 and 18:1 derivatives used in the previous study, in which a mixture of these common fatty acids was not analyzed (12,13). An isocratic mobile phase acetonitrile/isopropanol/water was separated successfully into two fatty acid derivatives. All derived fatty acids were separated from the major peaks of excess and decomposed reagents which were eluted faster than the reactive products. Though the 22:5 (n-3) derivative was not separated from the peak of 20:4 (n-6) with the present HPLC system, good separation of other derivatized fatty acids which usually occur in natural tissues was obtained. The retention time of each fatty acid derivative increased with increasing chain length and decreased with increasing degree of unsaturation of fatty acids.

A calibration graph was prepared by using the 17:0 ADAM ester which was used as the internal standard (Fig. 2). A linear relationship between the peak area and the amount of the

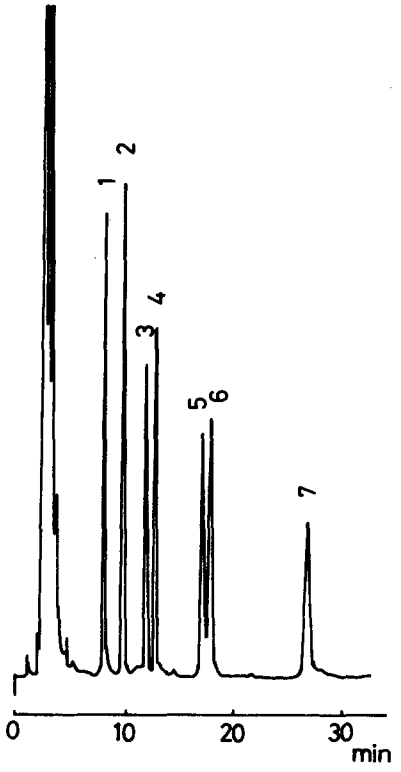

FIG. 1. Separation of the fatty acid ADAM esters mixture. The solvent system was acetonitrile/isopropanol/water (90:9:1, v/v/v). The flow rate was 1.2 ml/min. The fatty acid ADAM esters were resolved into 22:6 (No. 1), 20:4 (No. 2), 18:2 (No. 3), 22:4 (No. 4), 18:1 (No. 5), 16:0 (No. 6) and 18:0 (No. 7).

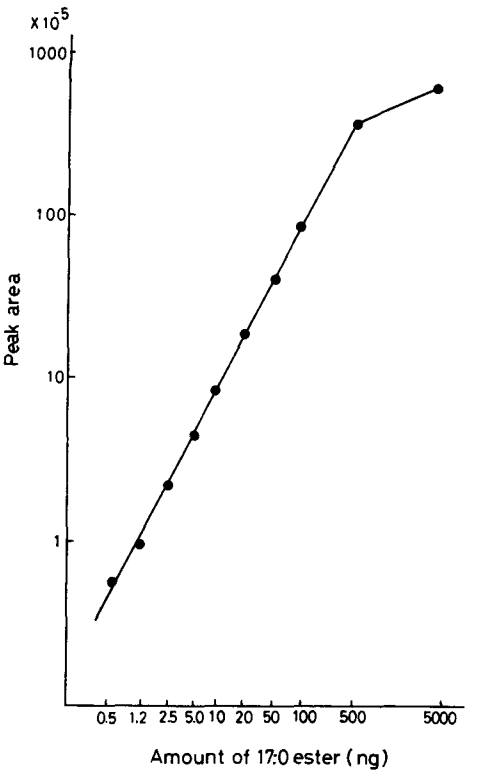

FIG. 2. Relationship between fluorescence intensity (peak area) and the amount of ADAM ester of heptadecanoic acid.

17:0 derivative was obtained in the range of 0.5-500 ng. ADAM has almost the same sensitivity as other fluorescent labelling reagents, such as 4-bromomethyl-7-methoxycoumarin (10) and 9-aminophenanthrane (11), and is 10 to 100 times more sensitive than reagents showing UV absorbance (5-9). These results indicate that the detection of free fatty acid ADAM esters at pmole level has become possible; thus, low levels of free fatty acids in biological tissues can be quantitated accurately.

In order to investigate the applicability of the present method at the cellular level, the free fatty acid ADAM esters of resident and activated alveolar macrophages were separated and quantitated by reverse-phase HPLC (Fig. 3). Table 1 shows the amounts of free fatty acids of alveolar macrophages. Alveolar macrophages (10^6 cells) contained approximately 2 μg of free fatty acids. As found in other mammalian tissues, 20:4 was quite a minor fatty acid in the free fatty acids fraction, representing only 0.8% of the total free fatty acids. The most predominant free fatty acid was 16:0, accounting for almost 50% of the total free fatty acids. No significant differences in the composition of free fatty acids were found with the ADAM esters separated by HPLC compared with that of fatty acid methyl esters determined by GLC.

Table 2 shows the composition of fatty acids which were esterified in glycerophospholipids, triacylglycerol and diacylglycerol. Triacylglycerol and diacylglycerol were composed predominantly of palmitic acid, which accounted for 53% and 43%, respectively. On the other hand, high percentages of unsaturated fatty acids, such as 18:1, 18:2 and 20:4, were found in phosphoglycerides. The proportions of unsaturated fatty acids in glycerophospholipids were significantly higher than those in the free fatty acid pool. The proportion of 20:4 in the free fatty acid pool was quite small (0.8%), and a significant proportion of 20:4 was found in the esterified fatty acids of glycerophospholipids. This result indicates that 20:4 was preferentially esterified in glycerophospholipids rather than

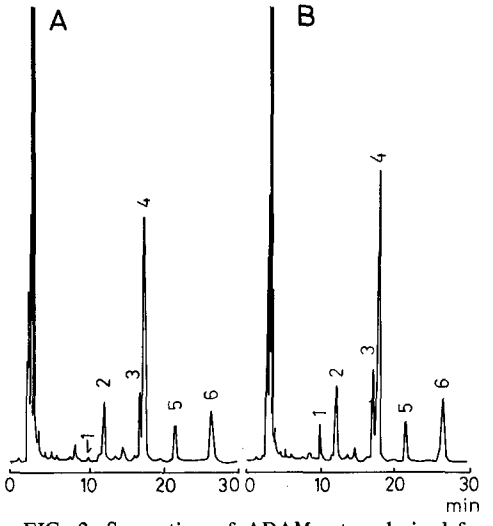

FIG. 3. Separation of ADAM esters derived from free fatty acids of resident (A) and activated (B) alveolar macrophages by reverse-phase HPLC. Alveolar macrophages (2×10^6 cells) were stimulated with opsonized zymosan in the presence of cytocharasin B for 1 hr. Total lipids were extracted from alveolar macrophages and fractionated into free fatty acids by TLC. The purified free fatty acids were derivatized to ADAM esters and resolved into 20:4 (No. 1), 18:2 (No. 2), 18:1 (No. 3), 16:0 (No. 4), 17:0 (No. 5) and 18:0 (No. 6). The heptadecanoic acid ADAM esters were used as the internal standard.

other fatty acids.

Table 3 shows the changes in the amounts of free fatty acids in alveolar macrophages after the challenge with opsonized zymosan as a function of time. The amounts of 20:4 increased gradually in activated macrophages. Opsonized zymosan produced an approximately 8-fold increase in the amount of 20:4 at 1 hr stimulation. Small but significant increases (1.5-fold) also occurred in the amounts of 16:0, 18:0, 18:1 and 18:2. These results show that the release reaction of fatty acids in activated alveolar macrophages was not selective for 20:4. Albert and Snyder demonstrated that the release of [^{14}C] 20:4 from rat alveolar macrophage glycerophospholipids was specifically enhanced by the treatment of prelabelled cells with zymosan or A 23187, whereas the release of radioactive 16:0, 18:1 and 18:2 was not increased above the control levels (17). We also confirmed these results using activated macrophages prelabelled with [^{14}C] 18:1, [^{14}C] 18:2 and [^{3}H] 20:4. While a small increase in the release of radioactive 18:1 and 18:2 was observed, it was considerably smaller than that seen with [^{3}H] 20:4 (data not shown). It has been shown that selective liberation of radioactive 20:4 from glycerophospholipids occurred in platelets (18), perfused kidney (19), lymphocytes (20) and fibroblasts (21), compared with other radioactive 2-acyl fatty acids when these cells were activated by specific stimulants. One possible explanation for this discrepancy between measurement of absolute mass and that of radioactivity of liberated free fatty acids is that "sensitive pools of stimulants" were preferentially labelled by radioactive 20:4, but other major 2-acyl fatty acid pools were not labelled. Radioactive 20:4 enters pools from which it can be released by stimulants. However, radioactive 18:1 and 18:2 do not directly enter the "sensitive pools of stimulants." Hsue et al. demonstrated the heterogenous pools of fatty acids. One is the specific release of 20:4 stimulated by bradykinin and the other is the non-

TABLE 1

Amounts of Free Fatty Acids of Resident Alveolar Macrophages

	16:0	18:0	18:1	18:2	20:4	Total
ng/10^6 cells	1099 ± 74	427 ± 31	353 ± 15	240 ± 28	17 ± 2	2136 ± 143
			Weight %			
HPLC[a]	51.4 ± 0.6	20.0 ± 0.4	16.5 ± 0.2	11.2 ± 0.7	0.8 ±0.6	
GLC[b]	52.9 ± 3.3	17.9 ± 1.1	14.8 ± 1.2	9.4 ± 1.4	–	

The values are expressed as the amounts (ng) of free fatty acids in 10^6 macrophages and are mean values ± SD (n=4).

[a]The values are the mean percentages of total free fatty acids determined by fluorometric HPLC.

[b]The values are the mean percentages of total free fatty acids determined by GLC after methylation with diazomethane.

TABLE 2

Compositions (by percent) of Fatty Acids of Glycerophospholipids, Triacylglycerol and Diacylglycerol of Alveolar Macrophages

	16:0	18:0	18:1	18:2	20:4
Glycerophospholipids	21.1 ± 0.4	12.3 ± 0.5	28.4 ± 0.2	21.5 ± 0.1	16.6 ± 0.4
Triacylglycerol	52.8 ± 2.2	8.8 ± 1.6	23.1 ± 1.1	14.3 ± 0.6	0.8 ± 0.3
Diacylglycerol	42.3 ± 1.0	15.2 ± 1.8	21.8 ± 0.9	9.9 ± 0.5	10.7 ± 1.4

The mean percentages (weight %) ± SD were determined for different samples (n=3). The fatty acids were analyzed as methyl esters by GLC.

TABLE 3

Changes in the Amounts of Free Fatty Acids of Alveolar Macrophages

	ng/10^6 Cells					
Time	16:0	18:0	18:1	18:2	20:4	Total
0	1204 ± 44 (100)	437 ± 12 (100)	346 ± 10 (100)	249 ± 21 (100)	20 ± 4 (100)	2170 ± 60 (100)
5	1286 ± 120 (107)	450 ± 31 (103)	378 ± 16 (109)	260 ± 27 (104)	23 ± 6 (115)	2305 ± 132 (106)
15	1207 ± 45 (100)	471 ± 39 (108)	396 ± 44 (114)	240 ± 23 (96)	80 ± 7 (400)	2252 ± 67 (103)
30	1539 ± 39 (128)	582 ± 53 (133)	496 ± 15 (143)	269 ± 14 (107)	135 ± 8 (675)	2895 ± 79 (133)
60	1720 ± 97 (143)	650 ± 25 (149)	651 ± 50 (188)	334 ± 41 (134)	167 ± 14 (835)	3357 ± 220 (155)

Alveolar macrophages were stimulated with opsonized zymosan in the presence of cytocharasin B for designated time periods. Free fatty acids were separated from the total lipid extract and derivatized to ADAM esters.

The free fatty acids ADAM esters were separated and quantitated by reverse phase HPLC as described in Materials and Methods. The values were the means of separate experiments (n=3).

Numbers in parenthese show the percent of control at 0 time.

specific release of all fatty acids caused by ischemia (19).

The different results for liberation of nonlabelled and labelled fatty acids from glycerophospholipids indicate that it is quite important to measure the absolute mass of liberated fatty acids in order to understand completely the possible mechanism of phospholipase activity.

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COMMUNICATIONS

Red Blood Cell Tocopherol and Liver Tocopherol in Hyperlipemic Rats as Compared with Plasma Tocopherol

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ABSTRACT

In rats with hyperlipemia induced by Triton WR-1339, changes in tocopherol concentrations in plasma and RBC were compared with those in the liver and its subcellular fractions, microsomes and mitochondria.

After daily injection with Triton, plasma total lipids at 3 days and 7 days, respectively, showed elevations 6.5 times and 15 times as high as those in the control rats, and triglycerides showed the most predominant elevation.

With the hyperlipemia, the concentrations of tocopherol in RBC and the subcellular fractions decreased, as plasma lipids and plasma tocopherol increased, while no change occurred in tocopherol concentrations in liver homogenates. The changes in the ratio of tocopherol to total lipids in plasma coincided with changes in tocopherol concentrations in the RBC and subcellular fractions.

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INTRODUCTION

Red blood cells (RBC) are known to depend on the nutritional intake of vitamin E (1-5). We have attempted to investigate the validity of the RBC tocopherol level as a clinical index of vitamin E status, and reported that RBC tocopherol reflects the ratio of tocopherol to lipids in plasma and also the platelet tocopherol, both of which have been emphasized previously as the best indices for vitamin E status assessment (6-9). Additionally, RBC tocopherol, which is known to be localized in the membranes, was observed to decrease when plasma lipid level was extremely high, in spite of much higher plasma tocopherol levels (10). However, the availability and validity of the above indices, i.e. RBC tocopherol, platelet tocopherol and the tocopherol/lipid ratio in plasma, have not yet been confirmed by direct measurement of the tocopherol content of tissues or membranes.

This paper examines whether tocopherol concentrations in tissues or subcellular fractions change in parallel with the RBC tocopherol concentration as hyperlipemia is induced. Rat livers were chosen as a tissue model because it is known that tocopherol in this tissue exists as a membrane component (11).

MATERIALS AND METHODS

Animals and Diets

The animals used were Wistar-strain male rats three weeks of age and weighing about 50 g. Their diet consisted of 36% corn starch, 25% vitamin free casein, 10% alpha starch of wheat, 8% powdered filter paper, 6% salt mixture, 5% granulated sugar, 2% vitamin mixture and 8% stripped corn oil (from Eastman Kodak Chemicals). The salt mixture provided: K, 692 mg; P, 579 mg; Ca, 411 mg; Na, 270 mg; Mg, 86 mg; Fe, 41 mg; Zn, 0.4 mg; Mn, 1.3 mg, and I, 7.7 mg per 100 g of diet. The vitamin mixture provided of vitamin A, 1,000 IU; D_2, 200 IU; E, 2.0 mg; B_1, 2.4 mg; B_2, 8 mg; B_6, 1.6 mg; B_{12}, 0.001 mg; C, 60 mg; K, 10.4 mg; biotin, 0.04 mg; folic acid, 0.4 mg; Ca-panthenate, 10 mg; para-aminobenzoic acid, 10 mg; niacin, 12 mg; inositol, 12 mg, and choline-Cl, 4,000 mg per 100 g of diet. Vitamin E was provided as all-rac-alpha tocopheryl acetate. Feeding of the diet began four weeks before the examination.

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Production of Hyperlipemia

After 4 weeks, the rats were given a daily intramuscular injection of 300 mg/kg of Triton WR-1339 dissolved in 1 ml of n-saline. The injections were given for three or seven consecutive days. The control rats were given the same volume of n-saline.

Preparation of Liver and Blood for Tocopherol Assay

The liver was homogenized with 10 times its volume of 0.25M sucrose by a Tephron homogenizer. The homogenates were divided into two portions. From one portion, subcellular fractions of supernatant, mitochondria and microsomes were prepared by Hogeboom's method (12). The remaining portion was used for measurement of the total amount of tocopherol in the liver. In this examination only the micro-

somes and mitochondria were used. Heparinized blood was centrifuged at 1,000 × g for 10 min to obtain separate RBC and plasma. The packed RBC were washed with three 10-ml portions of n-saline. During the last washing, close attention was paid to ensure an exactly constant 1,000 × g for 10 min to obtain a consistently packed cell volume.

Tocopherol Assay

The assay used a slight modification of Abe's method (13). Briefly, the samples (0.5 ml) were placed in a centrifuge tube together with 1 ml of 6% pyrogallol solution in ethanol, 1 ml of all-rac-tocol solution (2 μl/ml ethanol) as an internal standard and 0.2 ml of 60% KOH. Then the tube was incubated at 70 C for 30 min for saponification.

After cooling, 2.5 ml of water and 5 ml of hexane were added to the tube, which then was shaken vigorously for 5 min. The mixture was centrifuged at 1,000 × g for 5 min, and a 4-ml hexane layer was removed and evaporated under nitrogen gas flow at 40 C. The residue was dissolved in 50 μl of hexane, and 20 μl were injected into the HPLC column. The instruments used in this study were as follows: HPLC, Shimadzu LC-5A (Shimadzu Co., Kyoto); column, Nucleosil-5NH2, incorporating a 4-mm × 50-mm precolumn and a main column of 4 mm × 150 mm; eluent, n-hexane/isopropyl alcohol (97:3, v/v); flow rate, 1.5 ml/min; detector, Shimadzu RF-530 fluorospectrophotometer (Ex. 298 nm, Em. 325 nm). Retention time was 2.39 min for alpha-, 3.44 min for beta-, 3.68 min for gamma-, 5.20 min for delta-tocopherol, and 5.96 min for tocol, respectively. In this study, only the values for alpha-tocopherol are given.

Determination of Plasma Lipids

Total cholesterol, phospholipids and triglycerides were measured by the methods of Allein et al. (14), Takayama et al. (15), and Bucolo et al. (16), respectively. Total lipids were estimated by summing the three major lipids.

RESULTS

The effects of three or seven days of injections with Triton WR-1339 on plasma lipids are shown in Table 1. Triglycerides showed the most prominent elevation of plasma lipids in the Triton-injected rats, the level being 50 times higher than that in the control rats after three days of injections and 130 times higher after seven days of injections. The plasma total lipid level rose to 6.5 times and 15 times the original level after three days and seven days of injection, respectively. As hyperlipemia increased, plasma tocopherol levels rose markedly, as shown in Table 2. However, the elevation rate of plasma total lipids exceeded that of plasma tocopherol, although the tocopherol increase was proportional to the increase in total lipids. No body weight differences were observed between the hyperlimemic and control rats.

In the RBC, there was a significant decrease, about 25%, in tocopherol concentrations after seven days of injections, but no marked change was observed after three days of injections.

In the liver homogenates, tocopherol concentrations did not change during the experimental period. On the other hand, tocopherol concentrations in the microsomal and mitochondrial fractions were decreased by 35% to 40%.

The ratio of tocopherol to lipid in plasma decreased with the development of hyperlipemia.

DISCUSSION

Bieri (17) conducted an in vitro experiment to examine the transport of radioactive tocopherol from plasma to RBC, the plasma having been obtained from hypolipemic and hyperlipemic rats, and showed that incorporation of tocopherol into RBC decreased with increased plasma lipid concentrations. On the basis of this finding and other observations of obese and hyperlipemic rats (18), he hypothesized that a low tocopherol concentration in the nonadipose tissues in obese rats, contrary to the highly elevated plasma vitamin E levels as compared with controls, is best explained as a result of competition between the extremely high concentration of plasma lipids and the membrane lipids of lean tissue.

For the purpose of our study on the evaluation of RBC tocopherol level as an index of vitamin E (19-22), it may be a particularly noteworthy finding that with the increased plasma tocopherol level accompanying hyperlipemia, a significant decrease in tocopherol concentrations occurred not only in the RBC, but also in both the microsomal and mitochondrial fractions of the liver. Although Bieri (17) showed that exchange of tocopherol between RBC membrane and plasma occurs in vitro in a passive manner, our finding in liver also indicates that the exchange of tocopherol may occur in a similar passive manner with partitioning being determined by the level of total lipids between plasma and intracellular components. This remains to be elucidated.

The finding that no change was observed in tocopherol concentrations in liver homogenates during the progress of hyperlipemia may be explained by the idea that the Triton injected

TABLE 1

Changes in Plasma Lipids after Intramuscular Injection with Triton WR-1339

Groups	N	Body weight (g)	Cholesterol (mg/100 ml)	Triglycerides (mg/100 ml)	Phospholipids (mg/100 ml)	Total lipids (mg/100 ml)
Before injection	5	231 ± 9	106 ± 27	16 ± 3	69 ± 19	190 ± 40
Triton injection						
3 days	6	235 ± 9	182 ± 49**	769 ± 455**	266 ± 47**	1244 ± 542**
7 days	6	233 ± 16	324 ± 59**	2133 ± 438**	491 ± 106**	2927 ± 697**
Control (saline injection)						
3 days	4	231 ± 10	121 ± 13	21 ± 5	65 ± 9	207 ± 31
7 days	4	237 ± 15	111 ± 24	18 ± 6	74 ± 18	196 ± 46

The rats were intramuscularly injected once a day with either 300 mg/kg Triton WR-1339 or the same volume of n-saline, and killed 24 hrs after the injections. Each value represents the mean ± SD. Difference from level before injection: ** $p < 0.01$. Statistical analysis was performed by Student's t-test. N represents animal numbers.

TABLE 2

Changes in Tocopherol Concentrations in Plasma, RBC and Liver after Intramuscular Injection with Triton WR-1339

		Tocopherol concentrations					
				Liver			
Group	N	Plasma (μg/dl)	RBC (μg/dl)	Homogenate (μg/g)	Microsomes (μg/g)	Mitochondria (μg/g)	Toc/lipid ratio in plasma
Before injection	5	614 ± 131	314 ± 24	20.4 ± 1.9	0.222 ± 0.014	0.130 ± 0.012	3.40 ± 0.90
Triton injection							
3 days	6	2458 ± 951	305 ± 27*	17.9 ± 3.6	0.141 ± 0.037**	0.085 ± 0.024**	2.05 ± 0.28**
7 days	6	5426 ± 2102	240 ± 22**	20.3 ± 3.9	0.142 ± 0.038**	0.079 ± 0.028**	1.96 ± 0.58**
Control (saline injection)							
3 days	4	621 ± 86	330 ± 33	20.7 ± 1.5	0.221 ± 0.025	0.144 ± 0.011	3.01 ± 0.78
7 days	4	623 ± 129	303 ± 29	21.7 ± 2.9	0.249 ± 0.031	0.135 ± 0.030	3.20 ± 0.86

Each value represents the mean ± SD. N represents animal numbers.
Difference from level before injection: * $p < 0.05$, ** $p < 0.01$.
Statistical analysis was performed by Student's t-test.

might inhibit peripheral lipoprotein lipase activity (23) which catalyzes VLDL to form LDL, and that the VLDL might then accumulate in the liver as a fat deposit including tocopherol. Thus, tocopherol determined in the total liver homogenates in this case may be in part assayed as the tocopherol in fat deposits other than in the biomembranes.

From this result, we know that RBC tocopherol determination may give a good estimate of the bioavailable tocopherol in the effective sites as well as the ratio of tocopherol to lipids in plasma (6).

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Phosphorylation of Chloroform Soluble Compounds in Plasma Membranes of Human Epidermoid Carcinoma A431 Cells

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ABSTRACT

This study investigated a possible role for the epidermal growth factor (EGF) receptor protein tyrosine kinase in phosphoinositide metabolism with plasma membrane vesicles from human epidermoid carcinoma (A431) cells. We found a novel chloroform-soluble product radiolabeled with [gamma-^{32}P] ATP that did not migrate from the origin in the thin layer system designed to separate the phosphoinositides, appeared as a single band of M_r = 3500 on polyacrylamide gels in the presence of dodecyl sulfate, had an ultraviolet absorbance spectrum with a maximum at 275 nm and stained with Coomassie dye. Based on these properties this phosphorylation product is referred to as a proteolipid. The ^{32}P label was not detected in phosphotyrosine [Tyr(P)], phosphoserine [Ser(P)] or phosphothreonine [Thr(P)] and was lost during acid or base hydrolysis. Phosphorylation of proteolipid was increased significantly by EGF, whereas phosphorylation of phosphatidic acid was decreased and labeling of phosphoinositides was unaffected. Thus, it appears that in A431 membranes the EGF receptor/kinase does not utilize phosphatidylinositol as a substrate, but does phosphorylate a membrane proteolipid.
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INTRODUCTION

Epidermal growth factor induces a number of rapid morphologic changes in human epidermoid tumor A431 cells. Within five min of EGF stimulation the cells, normally flat and polygonal in culture, undergo fluid pinocytosis and ruffle and extend filopodia in the presence of extracellular calcium (1) but become round in the absence of extracellular calcium (2).

Circumstantial evidence suggests that the protein tyrosine kinase activity of the EGF receptor may be involved in mediating the cellular responses. Rous sarcoma virus, which encodes a protein that also contains protein tyrosine kinase activity, induced a similar sequence of morphological changes in infected chick embryo fibroblast cells (3). A431 cells stimulated with EGF or infected with Rous sarcoma virus accumulate at least one product in common, a M_r = 34,000 cytosolic phosphoprotein (4-6). How a kinase activity may be related to the rapid morphological changes is unclear. Recent reports claim that phospholipids serve as substrates for protein tyrosine kinases (7,8). To clarify the possible role of phospholipid phosphorylation in the mechanism of stimulus response coupling, the present study examined the effects of EGF on the phosphorylation of chloroform soluble compounds in isolated plasma membranes of A431 cells.

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EXPERIMENTAL PROCEDURES

Materials

Reagents and supplies were purchased from the indicated companies: EGF (Sigma Chemical Co., St. Louis, Missouri), [gamma-^{32}P] ATP (New England Nuclear, Boston, Massachusetts), constant boiling HCl, ninhydrin (Pierce Chemical Co., Rockford, Illinois), ethylenediamine tetraacetate (EDTA), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (Fisher Scientific, Fairlawn, New Jersey), sodium dodecyl sulfate, polyacrylamide, other electrophoresis reagents (Bio Rad, Richmond, California), X-Omat AR film and cellulose thin layer plates (Kodak), silica gel thin layer plates (Analtech, Newark, Delaware). Solvents were all of reagent or spectral grade. Phospholipids used as standards were purified on immobilized neomycin columns as described by Palmer (9) and were authenticated by comparing mobility on silica gel chromatography with standards obtained from Sigma Chemical Co.

Methods

Plasma membranes were prepared from A431 cells by the method of Thom et al. (10), as described by Brautigan et al. (11). Chloroform soluble products were extracted from reaction mixtures using a modification of the procedure of Folch-Pi and Stoffyn (12) in which chloro-

form:methanol:HCl, 500:500:4, was used in place of chloroform:methanol, 1:1. The chloroform phase was washed with a solution of the same composition as the aqueous phase of the extractions saturated with chloroform. Chloroform extracts were evaporated to dryness in glass tubes. The residues were dissolved in chloroform and applied to silica thin layer plates. The chromatograms were developed in a one dimensional solvent system designed to separate phosphoinositides as described by Schacht (13). Phosphorylated products were detected by autoradiography, identified by comparison of mobility of standards visualized with iodine vapor and quantitated by scintillation counting of silica gel scrapings. In some experiments, origin material was eluted from the silica gel with chloroform/methanol/conc. HCl (250:500:3) (C/M) and used for other analyses. Plasma membranes, at a concentration of approximately 1 mg/ml, were incubated at 30 C with 20 mM HEPES, pH 7.0, 500 μM $MnCl_2$, 500 μM $MgCl_2$ and 500 μM [gamma-^{32}P] ATP in the presence of either no further additions, 2 μg/ml EGF or 50 μM ortho-vanadate. Reactions were initiated by the addition of plasma membranes to the reaction mixture and were terminated at the indicated times by addition of the first extraction solvent.

Thin layer chromatography was performed as described by Zimmer et al. (14). Phosphoamino acid analysis on acid hydrolyzates was carried out as described by Brautigan et al. (11). A Cary model 219 spectrophotometer was used to record the ultraviolet spectrum (235 nm - 330 nm) of the proteolipid dissolved in C/M. Polyacrylamide gel electrophoresis in the presence of dodecyl sulfate was performed as described by Laemmli (15). Protein was estimated as described by Bradford (16) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Identification of Chloroform-Soluble Phosphorylation Products

Plasma membranes from A431 cells were incubated for 10 min at 30 C in the presence of 20 mM HEPES, pH 7.0, 500 μM [^{32}P] ATP, 500 μm Mg^{2+} and 500 μM Mn^{2+}. Lipid products were extracted by a modified version of the procedure of Folch-Pi and Stoffyn (12) and separated as described by Schacht (13). There were four major products labeled, three which migrated with phosphatidic acid (PA), phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP_2), and a fourth product which remained at the point of application on the silica gel thin layer plate (Fig. 1). Label was distributed among these products as shown in Table 1.

TABLE 1

Distribution of ^{32}P Incorporated into Chloroform Soluble Products

Product	^{32}P (%)[a]	Effect of EGF (%)[b]
Origin	29 ± 2.7	+ 35 ± 4 (9)*
PIP_2	1.4 ± 0.1	+ 14 ± 7.6 (10)
PIP	69 ± 3.6	+ 1.4 ± 4.7 (10)
PA	2.6 ± 1.0	- 45 ± 7.5 (4)**

[a]Plasma membranes from A431 cells, at approximately 1 mg/ml, were incubated with 20 mM HEPES, pH 7.0, at 30 C in the presence of 500 μM [^{32}P] ATP, 500 μM $MgCl_2$ and 500 μM $MnCl_2$ for 10 min. Data are reported as percent of total label ± standard error of 4 separate experiments.

[b]Results are reported as percent change from control ± S.E. Number of experiments listed in parenthesis; *, $p<0.05$; **, $p<0.01$ by Student's t-test for paired comparisons.

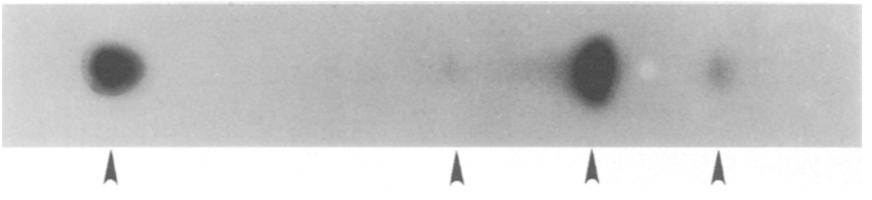

FIG. 1. Autoradiogram of silica gel thin layer separation of phosphorylated chloroform-soluble products from A431 membranes. A431 plasma membranes were phosphorylated for 10 min and products were extracted and separated as described in the text. The chromatogram is displayed sideways and the products are, from left to right, origin material, PIP_2, PIP and PA. R_f values: PIP_2, 0.33; PIP, 0.48; PA, 0.62.

The radiolabeled material that did not migrate from the origin in the thin layer chromatography system designed to separate phosphoinositides was eluted from the silica gel with C/M. The ultraviolet spectrum of the eluant has an absorbance maximum near 275 nm, a shoulder at 282 nm and a minimum at 262 nm. Such a spectrum is typical of protein in a nonpolar solvent. Lack of a peak around 265 nm indicates that the solution is relatively free of nucleotides.

The membranes were incubated with [^{32}P] ATP, extracted and the chloroform phase mixed with electrophoresis sample buffer. The resulting suspension was boiled for 30 min to evaporate the chloroform and was applied to a 15% polyacrylamide gel containing dodecyl sulfate. After electrophoresis, gels were dried and used for autoradiography. The major phosphorylation product migrated at the same position as the B chain of insulin with an estimated molecular weight of 3,500 (data not shown). When the complete reaction mixture was boiled in dodecyl sulfate and subjected to electrophoresis on a 15% polyacrylamide gel it also showed a major phosphorylation product migrating with an estimated M_r of 3,500 (Fig. 2) which stained with Coomassie blue. This band was excised from the gel, extracted with C/M and the extract applied to silica gel thin layer chromatography plates. Chromatograms contained a labeled product which did not migrate from the origin. These data suggest that the M_r = 3,500 band is a protein with covalently-bound phosphate that is the same as the origin material. As is customary for a product proteinaceous in nature and soluble in chloroform, the labeled material will be referred to as proteolipid. Other labeled material in the stacking gel, as well as at the top of the separating gel (see Fig. 2), was most likely the EGF receptor that would not migrate appreciably in a high percentage gel because of its relatively large size.

The material present at the origin after thin layer chromatography was eluted with C/M. Acid hydrolysis of this origin material for 2, 4 or 6 hr at 110 C was followed by high voltage electrophoresis at pH 1.9 and at 3.5 to analyze for phosphoamino acids. No Tyr(P), Ser(P) or Thr(P) could be detected. Nor were any peptides or other phosphorylated products detected by this analysis. Apparently the ^{32}P-phosphate was entirely released during acid hydrolysis. Label also was lost from the origin material by boiling for 5 min in 0.1 N NaOH. It remains unclear what type of bond attached the phosphoryl group to the proteolipid. Because of the acid and base lability of the protein-phosphate bond most of the usual phosphoamino acids are excluded, but an interesting possibility is that the product may be a cysteine-phosphate thioester (17).

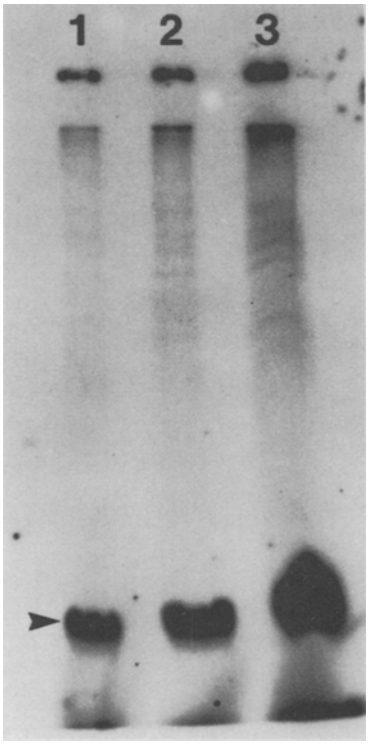

FIG. 2. Autoradiogram of ^{32}P labeled components of A431 cell membranes after electrophoresis in a 15% polyacrylamide gel in the presence of dodecyl sulfate. Membranes were phosphorylated as described in the text. After 10 min of reaction, the entire mixture was boiled in electrophoresis sample buffer. Lanes 1, 2 and 3 contained 10, 20 and 50 μl of sample. Material that did not enter the gel appears just below the numbers, and the arrowhead indicates M_r = 3,500. The gel was cut to remove the bromophenol blue tracking dye, a trace of [^{32}P] ATP is detected at the bottom edge. On gels of lower percentage the labeled products migrates so near the tracking dye it usually is not resolved from the excess nucleotide.

Effects of EGF on Labeling of Chloroform-soluble Products

Addition of EGF significantly increased the extent of labeling of the proteolipid. Incorporation of ^{32}P into proteolipid was time dependent, as shown in Figure 3, with maximal labeling within 5 min. The effects of ortho-vanadate, an inhibitor of protein phosphotyrosine phosphatase (18) and membrane Na^+/K^+-ATPase (19) also were examined. It was thought that if stimulation of a protein tyrosine kinase activity

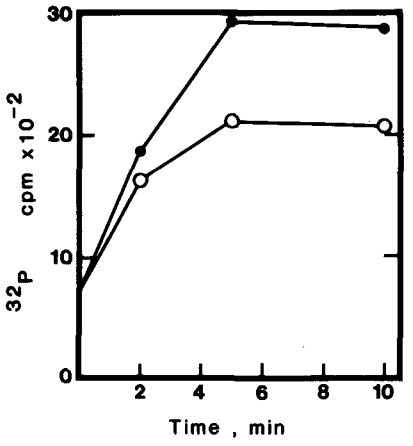

FIG. 3. Time course of incorporation of ^{32}P into proteolipid in A431 plasma membranes incubated with [^{32}P]ATP, Mn^{++} and Mg^{++}. Phosphorylation reactions were carried out in the presence (•) or absence (○) of EGF. Values are the averages of 4 separate experiments.

by EGF (20) is responsible for changes in phosphorylation of phospholipids, inhibition of protein phosphotyrosine phosphatase present in the membrane preparation might mimic the hormone-induced changes.

Table 1 also shows that EGF, which stimulates a protein tyrosine kinase activity in these membranes more than 3-fold, significantly increased ^{32}P accumulation in the proteolipid, decreased ^{32}P accumulation in PA but had no effect on PIP and PIP_2. In intact A431 cells, EGF was found to stimulate ^{32}P incorporation into phosphatidylinositol (PI) but only at times of an hour or more, attributed to PI turnover (21). Ortho-vanadate did not mimic the effects of EGF but increased ^{32}P incorporation into all four phosphorylation products. However, only the increases in PIP and PIP_2 were statistically significant.

The data indicate that EGF stimulation of the receptor/kinase is not associated with increased phosphorylation of diacylglycerol, PI and PIP. It is intriguing to consider that phosphorylation of proteolipid may be a link between binding of EGF to its receptor and physiological effects on A431 cells.

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Decreased Phosphatidylcholine in the Lung Fluid of Patients with Sarcoidosis

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ABSTRACT

Surfactant decreases the immune response of lymphocytes. Pulmonary sarcoidosis is a disease characterized by an increased number and activity of lymphocytes in the lung. We measured the lipids and lymphocytes retrieved from the lung by bronchoalveolar lavage. Thirteen patients with active pulmonary sarcoidosis had a significantly higher percentage of lymphocytes (18 ± 21%, mean ± standard error of the mean) than 10 control subjects (4 ± 1.9%, $p < 0.01$). Using an external marker, we found the absolute amount of disaturated phosphatidylcholine to be higher in the control group (174 ± 17.4 μg/ml lung fluid) than in the sarcoid group (91 ± 1.9 μg/ml of lung fluid, $p < 0.002$).
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INTRODUCTION

Disaturated phosphatidylcholine (DSPC), the major phospholipid in the lung and bronchoalveolar lavage (BAL) fluid, has been shown to suppress lymphocyte proliferation (1). Surfactant abnormalities have been noted in many disease states including infant (2) and adult (3) respiratory distress syndrome, acute lung injury (4) and pneumonia (5). A frequent abnormality of surfactant in disease is a decrease in the amount of DSPC present and changes in the fatty acid composition of phosphatidylcholine (PC) (2,4). Since DSPC may be an important immunoregulant in disease states, decreases in DSPC may be associated with unchecked lymphocyte activation and proliferation.

In pulmonary sarcoidosis, T-lymphocytes are sequestered in the lung and activated (6). We measured the amount of DSPC in the BAL fluid of patients with active pulmonary sarcoidosis. They showed a decrease in the amount of DSPC in the BAL fluid compared to a control population.

METHODS

Patients with sarcoidosis were recruited from those seen by the pulmonary service of the University of Cincinnati Medical Center. A control group consisted of normal volunteers. All subjects gave written consent to a protocol approved by the Human Research Committee of the University of Cincinnati.

Lavage was performed in the manner previously described (7). A flexible fiberoptic bronchoscope was wedged in either the lingula or the right middle lobe, and a total of 100 to 240 ml of normal saline with a known concentration of methylene blue was introduced and withdrawn with a handheld syringe. The aspirated fluid was pooled and its total volume noted. An aliquot was taken for cell analysis. The fluid was then centrifuged at 200 rpm for five min to remove cellular debris. By measuring the concentration of methylene blue in the introduced and aspirated fluid, the dilution of aspirated bluid by lung fluid could be calculated. We report our results per ml of lung fluid.

Albumin was measured in the supernatant using the rate nephelometry technique using an antibody specific for human albumin (8). A cell differential count was performed on a Wright-Giemsa stained slide of cells prepared from the BAL fluid by cytocentrifuge (Cyto-spin, Shandon, Pittsburgh, Pennsylvania).

Lipids were extracted from the BAL fluid using a modification of the technique of Folch et al. (9). Two ml of lung fluid were mixed thoroughly with chloroform:methanol (2:1, v/v) and 25 μg of dinonadecanolphosphatidylcholine used as an internal standard. The mixture was centrifuged, and the bottom layer was dried at 55 C under a stream of nitrogen. Lipids were extracted for measurement of either DSPC or PC.

DSPC measurement: A quantitative measurement of DSPC was made using a modification of the technique of Mason et al. (10). The lipid extracted from 2 ml of BAL fluid was mixed with 500 micrograms of osmium tetroxide for 15 min, dried at 50 C and the residue dissolved in chloroform:methanol (20:1, v/v). A column was prepared by placing 800 mg of alumina oxide (Fisher Scientific, Pittsburgh, Pennsylvania), activated for one hr at 110 C, on a glass wool plug in the neck of a 9-inch Pasteur

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pipette. The neutral lipids not reacting with osmium tetroxide were eluted with 10 ml of chloroform:methanol (20:1, v/v). The DSPC was then eluted with 5 ml of chloroform: methanol:7 M ammonium hydroxide (70:30:2, v/v/v). This solution was dried at 50 C under a stream of nitrogen and resuspended for subsequent fatty acid analysis.

PC separation: The lipid extracted by the Folch method was resuspended in 20 μl of chloroform and transferred to a thin layer chromatograph plate of silica G (Supelco, Inc., Bellefonte, Pennsylvania) prepared by incubation at 110 C for 1 hr immediately prior to use. The lipid was chromatographed using a solvent mixture of chloroform:methanol:water (95:34: 4, v/v/v) to separate PC (2). Standards of sphingomyelin, phosphatidylethanolamine and PC were run with every plate. After thin layer chromatography (TLC), the plates were developed with bromothymolblue spray reagent. The spot corresponding to PC and sphingomyelin was measured in length and width; the area of the spot is related to quantity (11). The spot corresponding to PC was scraped off and the lipid redissolved for subsequent fatty acid analysis.

Fatty acid analysis: The samples were evaporated to dryness. The fatty acids were released by incubating each sample in 0.5 ml of methanolic potassium hydroxide (15 g KOH in 100 ml of methanol) for 30 min at 65 C. After cooling to room temperature, 0.7 ml of 1M phosphoric acid and the fatty acids were extracted by 5 ml of hexane and analyzed with a gas chromatograph on a 6′ × ¼″ glass column packed with pretested 10% silar-10C on 100-120 mesh gas chrom Q (Supelco, Inc., Bellefonte, Pennsylvania) (12). A sample of 4 μl of the final trimethyl (triflurotolyl) ammonium hydroxide extract was mixed with 4 μl of methyl acetate and injected into the chromatograph (HP 5790A, Hewlett Packard, Avondale, Pennslyvania). Peak areas were measured with an electronic digital integrator (HP 3390A, Hewlett Packard, Avondale, Pennsylvania). The peak corresponding to methyl nonadeconal was used as an internal standard to calculate the concentrations of the fatty acids. In addition, the total amount of fatty acids for PC and DSPC were calculated.

Disaturated phosphatidylcholine (Sigma Chemical Co., St. Louis, Missouri) was diluted in chloroform and 0.9% sodium chloride (1:20, v/v) in serial dilutions from 500 mg to 50 mg per ml and then extracted as above for the quantity of disaturated phosphatidylcholine. Quantitative analysis of the fatty acid (palmitic acid) was performed and an excellent correlation between quantity of DSPC in the original volume and the final amount measured by the gas chromatography for a total of 13 separate runs ($r = 0.98$, $p < 0.001$). We therefore report all our fatty acid quantities in relation to the internal standard dinonadecanoylphosphatidylcholine which had been added in known amount prior to extraction.

RESULTS

Thirteen patients with sarcoidosis were studied. All cases had a clinical status and chest roentgenogram consistent with sarcoidosis as well as a skin, mediastinal lymph node, or transbronchial lung biopsy showing noncaseating granuloma. Nine of the 13 patients were female. No patient was on steroid therapy at the time of the study. The mean age of the sarcoid group was 32, with a range of 21 to 45. Eight of 10 subjects in the control group were male. The mean age of the control group was 31, with a range from 24 to 52.

The cellular analysis of the BAL fluid showed the predominant cell of the control group was the macrophage (95 ± 1.7%, mean ± S.E.M.) with only 4 ± 1.9% lymphocytes. In the sarcoid group, there was a significantly higher percentage of lymphocyte (18 ± 21%, $p < 0.01$) with only two patients having less than 10% lymphocytes in the BAL fluid. The total number of cells in the sarcoid group ($3.58 \pm 0.842 \times 10^7$ cells) was significantly higher than the control group ($1.82 \pm 0.193 \times 10^7$ cells, $p < 0.02$).

The dilution of introduced fluid by lung fluid was similar in the control group (0.16 ± 0.039) and the sarcoid group (0.20 ± .029). However, the amount of albumin found in the sarcoid patients (650 ± 153 μg/ml lung fluid) was significantly higher than in the control subjects (200 ± 23 μg/ml lung fluid, $p < 0.05$).

Analysis of the amount of DSPC showed that the total amount for the control group was 173.9 ± 14.75 μg/ml of lung fluid. In the sarcoid group, there was significantly less DSPC, 91.4 ± 10.95 μg/ml of lung fluid ($p < 0.002$).

Evaluation of the thin layer chromatography plates showed that the major phospholipid was phosphatidylcholine. The spot corresponding to PC was prominent for both groups, and the area corresponding to PC was always more than twice the area corresponding to sphingomyelin. There was a small, but similar spot, for phosphatidylethanolamine for both groups.

Analysis of the fatty acids in the PC spot showed that the major fatty acids were palmitic

(16:0), stearic (18:0), oleic (18:1) and linoleic (18:2) acids. The relative ratio per mole of each acid is shown in Table 1. The most noteable difference is the smaller percentage of stearic and oleic acid in the sarcoid patients.

CONCLUSION

This study demonstrated a smaller amount of DSPC in the alveolar space of patients with sarcoidosis compared to a control group of similar age. There was no discernable difference in the BAL findings in males and in females. Ansfield and Benson have shown that DSPC is a major immunosuppressant in surfactant (1). Although other phospholipids may be similarly immunosuppressant, because DSPC is the major phospholipid in BAL fluid, a decrease in DSPC may have significant immunologic consequences.

Since sarcoidosis is a disease mediated by lymphocyte proliferation and propagation in the alveolar space, DSPC is an important modulator. Our sarcoid patients had a higher percentage of lymphocytes in the BAL fluid, which is associated with active pulmonary disease (6). The decrease in DSPC in the BAL fluid may increase the lymphocyte activation and proliferation in the lung. Another possibility is that the increased lymphocyte activity may have blocked DSPC release from the Type II pneumocyte.

The alteration in DSPC seen in patients with sarcoidosis may be due to direct damage to the type II alveolar cell, that cell responsible for production, storage and release of surfactant. Acute lung injury has been shown to cause changes in surfactant in both quantity and quality. Ryan et al. have documented the sequence of changes seen in phospholipids, specifically PC, in the lungs of dogs after exposure to N-nitro-N-methylurethane (4). This toxin causes an acute alveolar injury with a pattern similar to the adult respiratory distress syndrome. The PC in the BAL fluid during the recovery of the dog has a higher percentage of palmitic acid than the pre-injury PC, with a corresponding decrease in the amount of stearic and oleic acids. A similar sequence of events has been noted after lung injury due to bleomycin and oxygen (13,14). Our patients with sarcoidosis had a relative increase in the amount of palmitic acid and fall in stearic and oleic acids.

There are some states where DSPC is not the only surface active agent. In patients with allergic alveolitis, there appears to be a relative and absolute fall in the amount of PC with an increase in the amount of phosphatidylethanolamine (15). In this study of patients with sarcoidosis, no change in the amount of phosphatidylethanolamine was found.

This is the first report of the absolute amount of DSPC in lung fluid from normal subjects and from those with sarcoidosis. Low et al. (16) reported a higher percentage of palmitic acid in PC recovered by BAL from normal subjects. This difference may be due to differences in lavage technique or sample size.

In conclusion, we found a decrease in the amount of DSPC in the sarcoidosis group. The changes in the phospholipids were similar to those seen during the recovery phase of acute lung injury. The significance of decreased surfactant in a disease characterized by lymphocyte-induced damage is yet to be characterized.

ACKNOWLEDGMENTS

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TABLE 1

Major Fatty Acid Composition of Phosphatidylcholine

	Palmitic acid 16:0	Stearic acid 18:0	Oleic acid 18:1	Linoleic acid 18:2
Control patients % of total fatty acids	62.1 ± 9.1[a]	15.8 ± 1.6	17.3 ± 5.2	4.9 ± 1.3
Sarcoid patients % of total fatty acids	77.3 ± 10.3[b]	7.4 ± 1.4[b]	12.2 ± 2.5[b]	3.2 ± 0.7[b]

[a]Mean ± S.E.M.

[b]Differs from controls, $p < 0.01$.

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Symposium

Papers from the symposium on
Analyses by Iatroscan TLC/FID System
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An Overview of the Iatroscan

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In comparison with other chromatographic techniques, TLC/FID (thin layer chromatography/flame ionization detection) is a relative newcomer, following closely, as it clearly does, on the heels of conventional TLC (thin layer chromatography), HPLC (high performance liquid chromatography), GPC (gel permeation chromatography) and GC (gas chromatography). At this juncture I think it would be highly appropriate to inject a qualifying statement as a precursor to what follows. It would be a mistake to table each and every individual feature manifest in those four chromatographic techniques in order to make a comparative study with TLC/FID, except for the purpose of establishing, for each discipline, recognition of its proper place in the analytical pecking order. However, where TLC/FID has no viable claim to usage is in the field of qualitative analysis and fluorescence techniques which are, respectively, economically and uniquely performed by conventional TLC plate methods. In essence, TLC/FID must be regarded as a complementary rather than a competitive element in relation to the classical chromatographic methods mentioned above.

The first TLC/FID development by Unilever, which culminated in the granting of patents in the late 1960s, was aimed strictly at quantifying lipids in fats and oils because of less than satisfactory TLC plate procedures. From a conceptual point of view, Unilever's project was a success, but from a practical standpoint, it was a near-failure because a lack of in-house technology relating to the design of precision mechanics, electronics and the modifications of silicas inhibited progress toward optimizing a system suitable for common laboratory usage. In parallel, but in total isolation from Unilever, Iatron Laboratories of Tokyo and Shionogi of Osaka (one of Japan's principal pharmaceutical companies) engaged with each other to develop independently for commercial purposes, a Thinchrograph (now known as the Iatroscan TLC/FID Analyzer), and Iatron eventually secured a license to manufacture the hardware. The main problem was to produce a suitably modified column substrate capable of withstanding high temperatures of a hydrogen flame for detection purposes while enabling substantial repetitive use of the coated substrate. This procedure would be quite unlike the one-time usage of a conventional TLC plate.

A technical solution to the column problem was found in a sintering process whereby silica gel or alumina is fused to a quartz support to form a particulate layer on the circumference of that support.

Essentially, what evolved was the coupling of TLC and a GC detector to produce a 'solid-phase' chromatographic system where predevelopment of samples could be made on a series of 10 inert micro-columns in the open. The sample components are located at fixed sites on the solvent-free substrate, according to their Rf value, and the columns are programmed to pass through a GC-type FID mechanically – hence the term 'solid-phase' is used to describe a direct substitute for the carrier gas system conventionally employed as a means of trans-

porting components through the closed column of a gas chromatograph.

Each column, known as a Chromarod, is a 1 mm solid quartz rod of 150mm in length onto which silica or alumina is sintered to a thickness of 75μ and upon which a TLC-like separation takes place. Solid samples and non-volatile high boiling substances, and non-fluorescing and non-UV absorbing materials can be separated and scanned rapidly. This suggests a wide application where suitable separation and detection conditions do not exist in GC, GPC, HPLC and TLC densitometry. In a word, the complementation of TLC/FID and the other methods is the feature by which its presence is ranked alongside the other methods.

From an application point of view, far from being identified with its origins, TLC/FID has spread extensively into fields where most organic materials require quantitative examination. Furthermore, group classification, compound typing, primary separation and fingerprinting render the technique a highly functional precursor to other more specific methods of sample analysis. This has been demonstrated in many industrial applications where rapid and repetitive sampling is needed for characterization and quantitation purposes. For example, in workplace environments, remarkably high recoveries of non-volatile organic materials such as particulate polycyclic aromatic hydrocarbons have been observed where their total presence remains inaccurately defined by GC and HPLC, either because of the loss of materials at the sample preparation stage or because of column adsorption effects.

In Europe and Canada, heavy emphasis has been placed on TLC/FID within the industrial sector, especially in respect of fossil fuels, lubricants, emulsions and surfactant analyses. In the case of fossil fuels this analysis is wide-ranging, from exploration core-drillings to production processes and to the investigation of refined oils to determine their in-service condition.

Many useful applications are to be found in the classification of materials according to their origin, or of products on the basis of their quality. This offers a broad spectrum to the TLC/FID user within the edible oil and related industries for unambiguous class identification of crude materials, base feedstocks through to pilot scale and regular production processes associated with such materials.

Generally, I would say that in Europe there has been a much greater patronage of TLC/FID by industrial users than has been the case in the U.S.A. but, in contrast, Canada has demonstrated a balanced demand between industrial and institutional use of the system—the emphasis in industry being mainly on raw material analysis. Also in Europe, inter-company laboratory comparisons are being made by which routines constantly are being refined and wherein the operational disciplines of TLC/FID dictate the absolute necessity to standardize the procedural work. Perhaps this meeting will stimulate objective comparisons among U.S. users who appear, in some cases, to show variables which are inconsistent with others' experience. Failure to conform to routines in the operation of the system undoubtedly will show up differences in manual expertise, thus giving some credence to the time-honored saying, "It's not what you do but the way that you do it."

Instrumentation, by virtue of the developer's design objectives, quite often achieves the distinction of performing its specific task. How well it exceeds performance expectations frequently can be attributed to the ingenuity of the operator or to the inbuilt flexibility of the system which later may be discovered by such an operator to perform beyond what was stated in the specification. I think it is true to say that the Iatroscan falls into such an instrument category and that there remains more to be discovered in terms of its potential use by contemplating the further practical application of the Chromarod itself. For example, it was recently used, non-chromatographically, in successful experiments to measure benzene solubles in workplace atmospheres and currently is being examined for the purpose of carrying separated TLC components into a mass spec. It is, I believe, quite reasonable to assume that there is a great deal more to be learned from TLC/FID than has been determined so far.

In conclusion, I am obliged to confuse matters slightly by stating that TLC/FID already is an obsolete generic term to describe the present state-of-the-art of combining a TLC Chromarod with a GC detector. In tandem with the FID, an FTID (flame thermionic ionization detector) nitrogen-specific detector will be presented at this meeting. Furthermore, an FED (flame emission detector) for sulphur detection is in development. Both will serve to illustrate the add-on flexibility of this hybrid technique in addition to the semi-automated multi-sample applicator system which promises to contribute a high degree of standardization and technical improvement to the precision and accuracy of the chromatography by removing another source of user variable—so essential if one is to concentrate on making the chemistry work rather than to seek a premature compromise in the computer 'massage parlor.'

[Received October 15, 1984]

A Specific Detector for Nitrogen and Halogen Compounds in TLC on Coated Quartz Rods

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ABSTRACT

A new detector has been developed for use with the Iatroscan TH-10 TLC/FID instrument–a "solid-phase" chromatographic system (SPC). This new detection system contains a unique stage of detection that is called a flame thermionic ionization detector or FTID. The FTID evolved from a similar device that recently has been used in gas chromatography. The basic concept of an FTID is that organic sample compounds are first decomposed by combustion in an H_2-air flame. The FTID does not sense any of the ionization produced directly in the flame but, instead, re-ionizes the combustion products by means of a unique thermionic transducer placed in the downstream effluent of the flame. The thermionic transducer ionizes specifically only those combustion products which are acidic or electronegative in chemical activity. In particular, organic samples containing nitrogen (N) or halogen atoms combust to products which are especially well detected with high sensitivity. Hence, the FTID adds to the Iatroscan a state-of-the-art detection stage which produces large signals for nitrogen and halogen compounds and negligible signals for hydrocarbon compounds.

Lipids 20:503-509, 1985.

INTRODUCTION

In the Iatroscan TH-10 chromatographic system (Iatron Laboratories, Tokyo, Japan. World distributors, Newman-Howells Assoc. Ltd., Winchester, U.K. U S. agent Ancal Inc., 1530 Bayview Heights Dr., Los Osos, CA 93402.), samples are separated by a TLC-type technique on small diameter quartz rods coated with a silica or alumina frit. Sample mixtures normally are spotted near one end of the rod, and chromatographic separation is achieved along the length of the rod by development in an appropriate solvent. The Iatroscan system then provides a novel means of quantitation by controllably moving the rod through the hydrogen-air flame of a flame ionization detector (FID).

The FID is a detection technique that originally was developed in the field of gas chromatography. Several other gas chromatographic detection techniques also use flames, and are therefore potentially applicable to the Iatroscan system. This paper describes such an application involving a detector which provides specific responses to compounds containing nitrogen or halogen atoms.

EXPERIMENTAL

Hardware and Electronics

Figure 1 shows a schematic diagram of the new Iatroscan detection system which consists of a series combination of an FID and FTID detection stage. The detector hardware is mounted onto a modified top cover of the Iatroscan. Installation of the new system simply involves removal of the existing FID collector and top cover and replacement by the modified top cover with the new detector system attached to it. As indicated in Figure 1, the new detec-

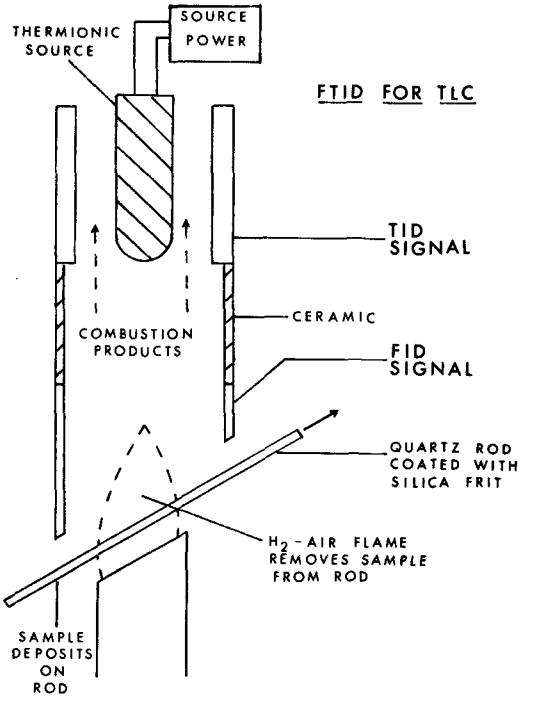

FIG. 1. Schematic diagram of the new flame thermionic ionization detector (FTID) of the Iatroscan system.

tion system includes a metal FID collector which is connected via a ceramic tube to the thermionic transducer. The entire detector structure can be adjusted in the horizontal and vertical direction to allow alignment of the detector system with the Iatroscan burner and Chromarods.

The mechanism of ionization in the flame ionization detector (FID) stage is a gas phase process. The high temperatures and chemically active species in the flame vaporize and decompose sample compounds from the Chromarods and simultaneously ionize them. A polarization voltage on the burner causes negative ionization formed to move to the FID collector where the signal is measured. As is well known, the FID produces a response to virtually all organic compounds.

In contrast to the FID, the mechanism of ionization operative in the FTID is a surface ionization process. The ionization in this stage occurs at a location well removed from the chemically active flame environment of the Iatroscan burner. The neutral products of sample decomposition flow downstream of the flame into the thermionic transducer where they impact the surface of a unique, electrically heated, ceramic coated thermionic source.

The thermionic source coating is impregnated with a cesium compound so that it is capable of emitting electrons when sufficiently heated. Thermionic ionization of combustion products occurs by a simple process of electron transfer from the hot thermionic source to impacting chemical species which are electronegative in chemical nature. Hence, unlike the FID, the FTID provides responses only to compounds that yield electronegative combustion products, and these are primarily compounds containing nitrogen or halogen atoms.

The FTID of the present system should not be confused with the NP-type thermionic detectors that have been popular in recent years in gas chromatography. The basic difference between an FTID and an NPD for GC is the chemistry of the gas phase environment immediately surrounding the hot thermionic source. In the FTID, samples are pre-decomposed in a flame which is well removed from the thermionic source. Hence, the FTID thermionic source resides in a gas environment comprised of hot unburned air, hot water vapor produced from the H_2-air combustion, and sample compound combustion products. In contrast, the thermionic source in an NPD resides in a very hot, highly reactive gaseous boundary layer comprised of dissociated hydrogen and oxygen molecules, as well as dissociated sample compounds. In the NPD, the state of dissociation of the H_2 and O_2 is maintained by operating the thermionic source at a very hot (i.e. 600-800 C) surface temperature. In the FTID, the operating temperature of the thermionic source usually is lower (i.e. 400-600 C). As a result of these comparisons, there is a distinct difference in response characteristics between an FTID and an NPD.

Precisely controlled electrical heating currents for controlling the temperature of the thermionic source are provided by an electronic power module packaged as a unit separate from the main Iatroscan instrument. The same power module provides a polarization voltage for the thermionic source so that negative ionization moves to a surrounding collector electrode, thereby producing a thermionic (TID) signal.

The new detection system for the Iatroscan, therefore, can provide a universal FID signal and a nitrogen (halogen) specific thermionic (TID) signal for each sample compound vaporized from the Chromarods. The FID signal is measured using the electrometer amplifier normally supplied in the Iatroscan instrument. The TID signal is measured with an additional electrometer module which is attached to the thermionic source power supply. The TID electrometer is a conventional GC-type electrometer having five ranges of amplification (10^{-12} A/mV to 10^{-8} A/mV) and a xl to x1024 attenuator. Using a dual-channel recording or integrating data display, both the FID and TID signals may be recorded simultaneously or separately.

RESULTS AND DISCUSSION

New Analytical Possibilities with the FTID

Nitrogen (or halogen) specific detection is an important new capability now available for the Iatroscan. The ability to obtain simultaneously both FID and TID signals is a further capability that opens up new possibilities for Iatroscan usage. Figure 2 shows the simultaneous recording of FID and TID signals for a variety of different chemical compounds spotted onto the Chromarods which have not been chromatographically separated. Since the quantities of these sample compounds differed widely, the relevant data are the ratios of FID to TID signals rather than the absolute magnitudes of each signal. Note that 'oil' samples produce very large FID/TID signal ratios because they are composed predominantly of hydrocarbons. In contrast, the sample of chloro- and nitrophenols produced a very low FID/TID ratio, thereby exhibiting the high FTID specificity to N or Cl compounds. The relatively low FID/TID ratios for urine and milk indicate that both these samples contain constituents which can be

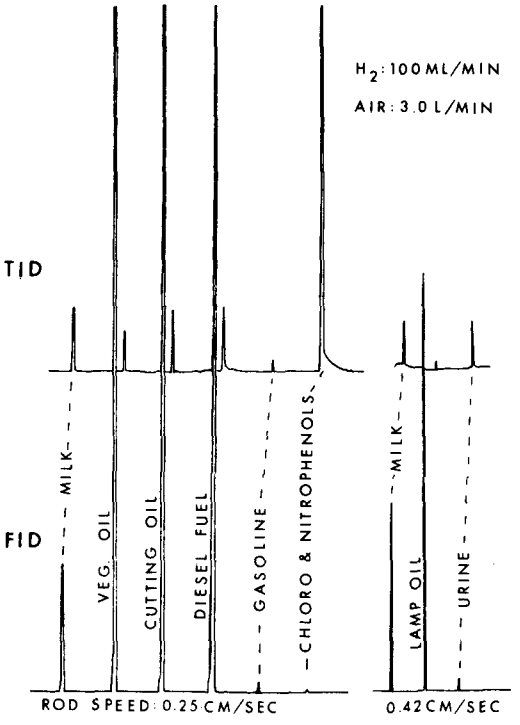

FIG. 2. Simultaneous FID and TID signal of a number of different chemical compounds spotted along a Chromarod.

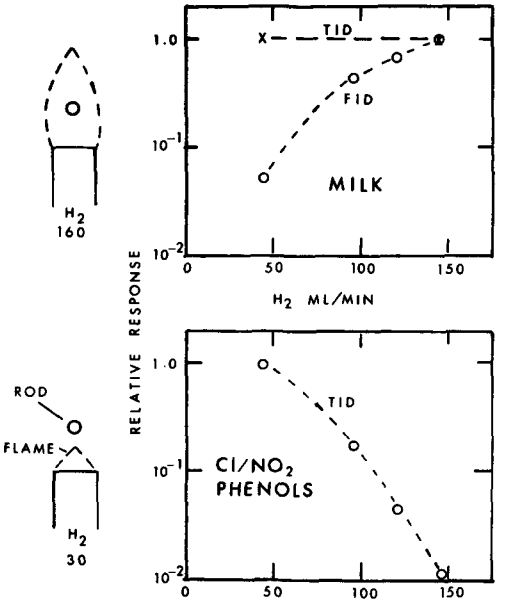

FIG. 3. The FID and TID detector response of milk and chloro- and nitrophenols to different H_2 flow rates.

detected specifically with the FTID. For example, in the case of the milk sample, the TID signal undoubtedly is correlated with the magnitude of milk protein because proteins are the principal N-containing compounds in the milk. With an element specific detector such as the FTID, therefore, there are the dual possibilities of measuring both total -N content of samples (i.e. samples spotted on Chromarods but not developed), and N-speciation of samples developed and chromatographically separated on the Chromarods.

Figure 3 illustrates that the new FTID system also offers the potential of obtaining data for a wide range of flame-Chromarod interactions. The FID and FTID stages of detection present two contrasting situations. In the FID, the flame is used for both sample vaporization and ionization, and the two processes are interdependent on the flame interaction with the Chromarod. In the FTID, the ionization stage of detection is well-removed from the sample vaporization stage, so that it becomes possible to retain a measurable ionization signal while the flame-Chromarod interaction is varied over a wide range. The principal way of varying the flame-Chromarod interactions is by varying flame size through variations in the H_2 supplied to the burner. The data in Figure 3 range from two extreme conditions as follows:

1. At H_2=160 ml/min, the flame encompasses the Chromarod completely, and the chemically-active flame species effectively remove all the sample compounds on the Chromarods.

2. At H_2=30 ml/min, the flame is very small and sample compounds on the Chromarods are subject to a more selective 'steam extraction' process of vaporization.

Figure 3 shows that FID signals diminish as expected with decreasing H_2 flow, but that TID signals can exhibit the opposite effect. In fact, for the phenol sample, there is a 100-fold increase in TID signal in varying H_2 from 150 ml/min to 50 ml/min. The new FTID stage of detection, therefore, offers not only element-specific detection for the Iatroscan, but also the possibility of selective vaporization of only certain components from the sample residing on the Chromarods. For example, a possible analysis scenario might begin with a 'steam extraction' and detection of N- or Cl-compounds at a low H_2 flow, and a re-run of the same Chromarod at higher H_2 flow for FID and TID detection of the residual sample left on the Chromarod.

Figure 4 shows more data on detection response versus H_2 flow. The data for caffeinated

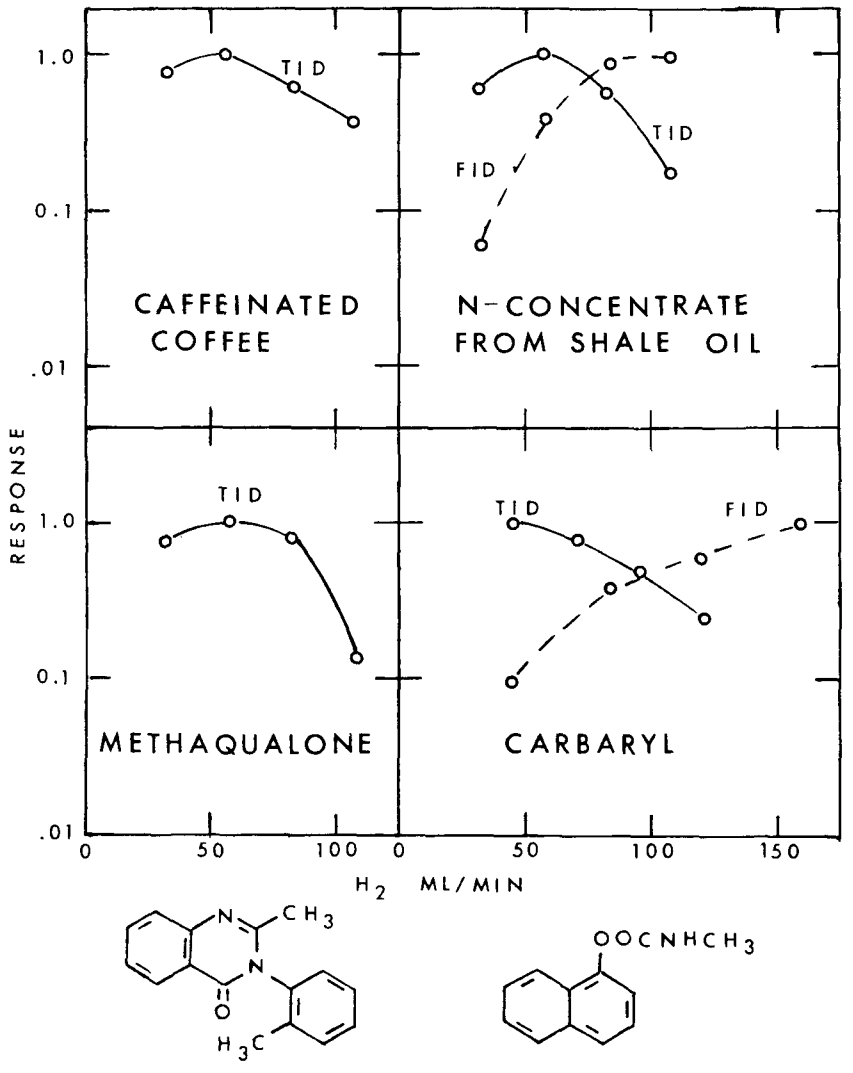

FIG. 4. The FID and TID detector response of several other selected compounds or complex mixtures to different H_2 flow rates.

coffee and an N-concentrate fraction from shale oil represent complex mixtures of sample compounds spotted onto the Chromarods but not chromatographically separated. The data on methaqualone (drug-of-abuse) and carbaryl (widely-used insecticide) represent single, pure compounds spotted onto the Chromarods. All these samples contain N-constituent compounds, and the molecular structures of methaqualone and carbaryl are indicated at the bottom of Figure 4. Note that the TID signal in all cases is substantially higher at H_2=50 ml/min than at the usual H_2 flow of 160 ml/min.

FID Improvements

The vertical position of the new detection system is set reliably relative to the burner and Chromarod by using a thin metal 'gauge' strip that is laid onto the top of the Chromarod rack in the Iatroscan instrument. When set, the bottom of the FID collector is 1 mm above the top plane of the Chromarod rack and approximately 2.5 mm above the Chromarod. This gives a substantially closer spacing of the FID collector to the Iatroscan burner than normally has been the case in the past. This new, closer

spacing results in a noticeable improvement in collection efficiency for ionization produced in the flame. The situation is illustrated schematically in Figure 5. Because the outer metal sleeve of the Iatroscan burner is at ground potential, there is a finite probability that lines of electrical force that begin at the polarized center of the burner will terminate at the outer sleeve of the burner without ever intersecting the FID collector. If the spacing between the collector and the burner is reduced, then more lines of electric force intersect the collector and the flame ionization collection efficiency is improved.

Data in Figure 6 illustrates the difference in response observed between a typical Iatroscan FID having the 'old' wide spacing, and the FID on the new detector structure which has a closer spacing of collector to burner. The test substance is carbaryl, which contains N and O atoms in addition to C and H. Sample amounts of 0.3 μg and 3.0 μg were measured with both FID versions. It can be seen that the newer FID spacing produced substantially larger peaks, and the ratio of the 3.0 to 0.3 peaks is approximately linear.

Both sets of FID data in Figure 6 were recorded on a data system display at 64mV full scale sensitivity. The 0.3 μg and 3.0 μg samples were spotted onto two different Chromarods, and the negative spikes exhibited in the two recordings are caused by electrical transient pickup from the electrical contact switches activated in the motor-driven Chromarod scans. The electrical transient pickup, therefore, is the ultimate limitation on FID detectability, rather than electronic noise originating within the electrometer.

Also shown for comparison in Figure 6 is the FTID response to a carbaryl sample. Note that the FTID sample is a 10-times smaller amount (0.03 μg) and a substantially larger peak height than the smallest sample detected by the FID. Hence, the FTID produces about 100 times greater sensitivity (i.e. signal-to-noise) than the FID, in addition to being very specific for nitrogen or halogen compounds.

Figure 7 illustrates another area of FID improvement that has been realized from the new, closer spacing of the FID collector. This improvement relates to variations in FID signal

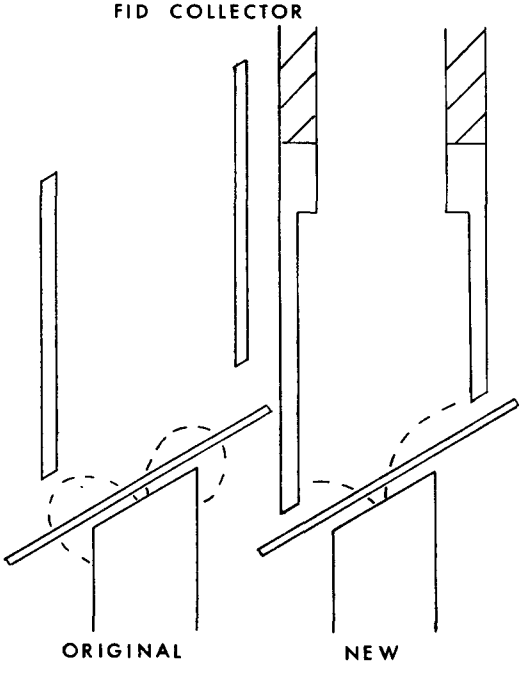

FIG. 5. Schematic diagram of the FID collector at two settings above the burner and the probable lines of electrical force.

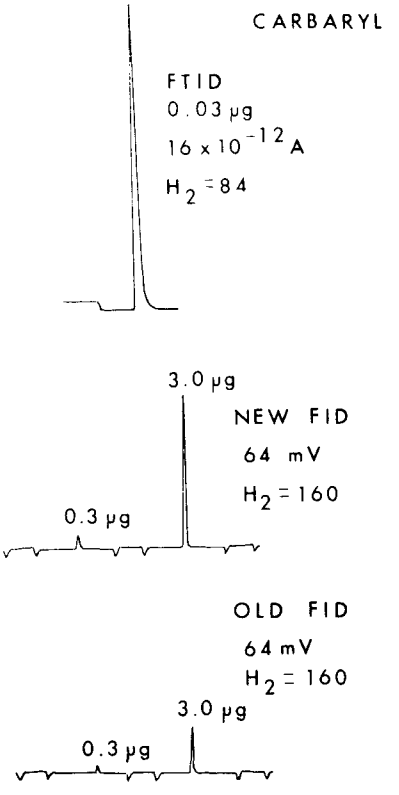

FIG. 6. Comparison of the FID signal response to the same amount of carbaryl at two separate settings of the FID collector, and to 1/100th the amount using the FTID.

as the H_2 flow is varied. With the older wide spacing of the FID collector from the burner, a H_2 flow of 160 ml/min evolved as the recommended standard for H_2. Decreases of H_2 from 160 ml/min were known to cause decreased FID signals as illustrated in the top graph of Figure 7. With the new, closer spacing of the FID collector, the FID signal in the bottom graph of Figure 7 does not change substantially until the H_2 flow drops below 100 ml/min.

The data in Figure 7 indicate that the new detection system opens up the possibility of using H_2 flows lower than 160 ml/min without sacrificing signal. This is a significant improvement because it also opens up the possibility of using slower Chromarod scan speeds. When the Iatroscan burner is operated at the usual H_2 flow of 160 ml/min and air flow of 2.0 l/min, the resulting large flame is almost hot enough to cause the Chromarods to overheat in the flame. In fact, with these previous standard flow rates, usually only fast Chromarod scan speeds could be used without rod overheating. With lower H_2 rates, corresponding lower rod scanning speeds also can be used, thereby providing greater flexibility in optimizing Iatroscan parameters to achieve the best possible analysis for a given sample.

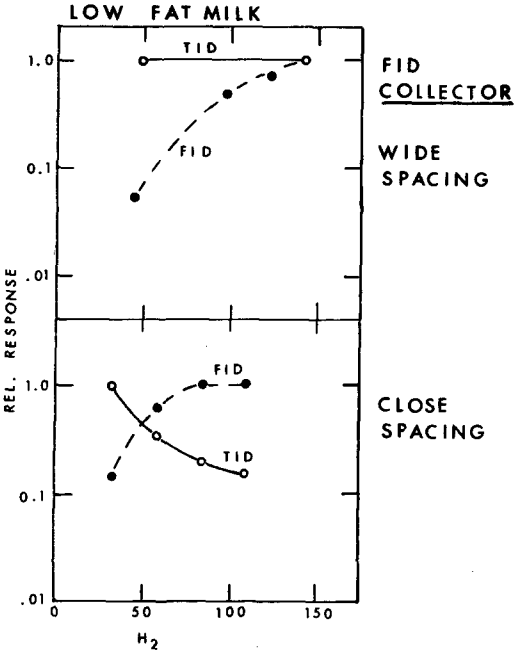

FIG. 7. Comparison of the FID and TID signal response of low milk fat to different H_2 flow rates at two separate settings of the collector.

Linearity of Response

The standard Iatroscan FID is known to exhibit response signals which often are not a pure linear function of sample concentration. One basic element affecting FID linearity is undoubtedly the fact that samples analyzed via the Iatroscan technique often are very large in sample amount. Hence, as various sample compounds volatilize from the Chromarod, the flame exhibits transitions from a hydrogen-fueled flame to a hydrogen-plus-organic-sample-fueled flame. In FID applications in gas chromatography, it is precisely in this same sort of transition region that GC-FIDs begin exhibiting non-linear effects.

In the FID data shown earlier in Figure 6, the new FID configuration exhibited a linear response for the sample Carbaryl at the 0.3 μg to 3.0 μg range. However, in the data exhibited in Figure 8, the new FID configuration exhibits a non-linear response for an unseparated milk sample. In Figure 8, the FID response is plotted versus sample amount on a log-log type of plot. If the response is linear with sample amount, then the data on this type of plot would define a straight line having a slope of 1.0 such as is indicated by the dashed line in Figure 8. As may be seen, the data, instead, defines a line of slope 1.2, meaning that sample response increases as the 1.2 power of sample amount for

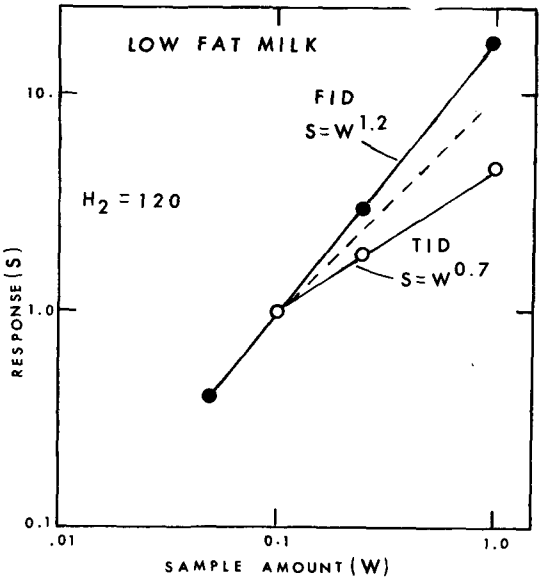

FIG. 8. Plot of the log of the FID and TID responses versus the log of the amount of sample (low fat milk) applied.

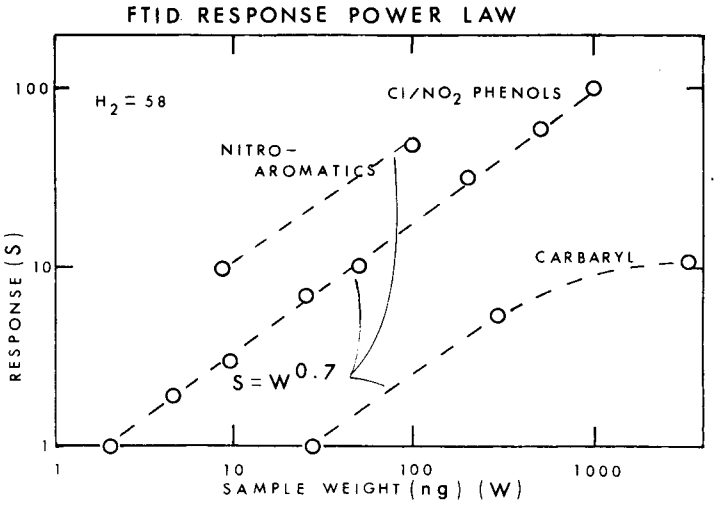

FIG. 9. Plot of the log of the FTID response versus the log of the amount applied for nitroaromatics, chloro- and nitrophenols and Carbaryl.

the 20-fold range of sample amounts illustrated in Figure 8. Although not linear, such a sample response nevertheless can be quantitated once the appropriate power law is established.

Also plotted in the graph of Figure 8 are the TID signals for various amounts of the milk sample. The FTID data exhibit a 'sub-linear' response vs sample amount, whereas the FID response was 'super-linear.' Figure 9 shows additional FTID data for other sample types and for a different H_2 flow than Figure 8. Most of these data are well represented by a response power law of 0.7 (or about 2/3), which suggests that this non-linear FTID response is the result of some fundamental physical/chemical mechanisms occurring in the system.

The FTID for TLC belongs to an expanding family of chemical transducers which use the thermionic ionization principle. For example, in gas chromatography there are available six distinctly different modes of detector response using simple variations of the same basic technology. Therefore, as the thermionic technology continues to expand, it is quite reasonable to expect that the FTID for TLC will benefit from an increased understanding of the basic technology.

SUMMARY

The new detection system for the Iatroscan offers the following features:

- Series combination of a flame ionization detector (FID) and a flame thermionic ionization detector (FTID).
- FID provides universal response to all organics.
- FTID provides specific responses to compounds containing nitrogen or halogen atoms.
- Simultaneous or separate FID and FTID signals for each sample compound.
- FID sensitivity of 10^{-7} gm or better.
- FTID sensitivity of 10^{-9} gm or better (for N-compound, Carbaryl) and N/C specificity of 10^3.
- FTID electrometer with 5 amplification ranges, 10^{-12} A/mV to 10^{-8} A/mV, and xl to x1024 attenuator for output signal.

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Improved Sample Application Methods for the Iatroscan

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ABSTRACT

A new method of sample application has been developed for Chromarods to give improved interrod and interrun repeatability. A rotary method using a small rig has been constructed to hold and rotate the rod as the sample is applied. Improved interrod repeatability of 0.5-0.7% standard deviation compared to 1.3-3.3% standard deviation using the Iatroscan recommended method has been achieved. *Lipids* 20:510-515, 1985.

INTRODUCTION

Conventional thin layer chromatography requires a wide variety of techniques to visualize the separated components. Although these can give considerable qualitative selectivity they also can be time consuming and may lead to missing some components if the appropriate visualization technique is not used. Another problem is the wide range of response factors obtained from different compounds which prevent even semi-quantification without proper standards. These problems are to a large extent overcome in the Iatroscan TH-10 analyzer. This instrument was developed from the work of Padley (1). The technique uses a special rod, a Chromarod, having the active silica or alumina thin layer sintered onto it. The separation takes place in this layer. Having separated the components and removed the developing solvent by drying it in a stream of nitrogen, the rods are placed in the detection apparatus. The separated components are detected by passing the rod under controlled conditions through the hydrogen/air flame ionization detector (FID). Thus, all combustible organic components are detected and the response per mole of the detector should, in principle, be roughly proportional to the carbon content of the compounds.

The accuracy of the Iatroscan has been investigated using standard lube oil blends. The results indicated that the instrument produced a non-linear response (2). Further work with standards, n-hexadecane and sorbitan trioleate, showed a similar non-linear response pattern.

Within BP (British Petroleum), an Iatroscan inter-center correlation program (2) showed poor intra-laboratory repeatability (ranging from 2 to 30%) which suggests that the technique, at best, should be considered as semi-quantitative.

One reason for the poor results was the way in which the sample was applied to the chromatographic rod. Until this investigation, the sample was applied as recommended by the Iatroscan manufacturer. A portion of the sample solution was applied to the rod as a discrete "drop" in one place using a capillary pipette. This is considered to be a rather "hit or miss" method of application, as the rod is circular and there is no certainty that the sample will spread out circumferentially during development. In addition, when the rods were taken from their development frame and put in the scanning frame it was difficult to ensure they were all oriented so that the sample application position was the same for all rods, relative to the flame profile. Also, combustion conditions may have altered from rod to rod.

A study was carried out to evaluate the repeatability of the Iatroscan technique using four different sample application methods. These methods were:

(i) Spot application using the traditional method with a disposable micropipette.

(ii) Spot application onto a stationary rod as in the recommended Iatroscan method, but using a micrometer operated syringe with a modified needle for more precise delivery.

(iii) Rotating the Chromarods while dispensing the sample, over a constant time period, from the micrometer syringe used in (ii) above, so that a sample is applied as a uniform band around the circumference of the rod.

(iv) Rotating the Chromarods while dispensing the sample as a fine aerosol spray using a propietary aerosol applicator.

Various surfactant samples and biological extracts were used in these investigations.

EXPERIMENTAL

Instrumentation

Sample analyses were carried out with an Iatroscan TH-10 TLC/FID analyzer (Iatroscan Inc., Tokyo). Chromarods SI and SII (Iatroscan Inc., Tokyo) were used in sets of 10. The sample was applied using either a disposable 1 μl micropipette (Camlab, Cambridge) or an Agla micrometer (Burroughs Wellcome, London) fitted with a modified Hamilton 10 μl micro-

syringe and with a drawn out polythene tip (ID 0.2 mm). Developing chambers for Iatroscan Chromarods were supplied by ACS, Luton. The rotary applicator rig (MKI) (Fig. 1) consisted of a synchronous 5 rpm 240 V motor (Crouzet) fitted to a base. The Chromarods were connected to the motor via a small piece of silicone tubing. At the other end, the rod passed through a PTFE "0" ring and was weighted with two small stainless steel washers. Additional aids used with the rig were a magnifying glass and syringe alignment guide.

The MKII rotating rig consisted of a portable Chromarod frame which could be used in all three stages of the Iatroscan operation, sample application, Chromarod development and FID scanning detection. The rig, shown in Figure 2, consisted of a synchronous 2 rpm 240 V motor (Crouzet) fitted with a drive shaft adapted to take a modified tape cassette drive roller. The Chromarod frame was secured onto a sliding carriage and the roller gently brought into contact with the end of the exposed Chromarod to effect rotation of each rod in turn. The sample was applied using a 10 μl Hamilton syringe (Camag, Switzerland) fitted in the Camag Linomat III (Camag, Switzerland).

Data was recorded and results calculated using a Hewlett-Packard 3390A integrator connected to the analogue output of the Iatroscan TH-10.

Materials

For TLC development of the Chromarods, redistilled Analar reagents (BDH) were used for the mobile phases. Surfactant and lipid samples were obtained from commercial suppliers and

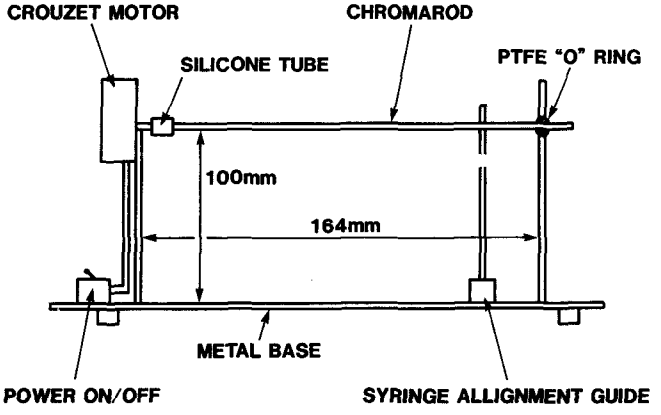


FIG. 1. Mark I rotary application rig.

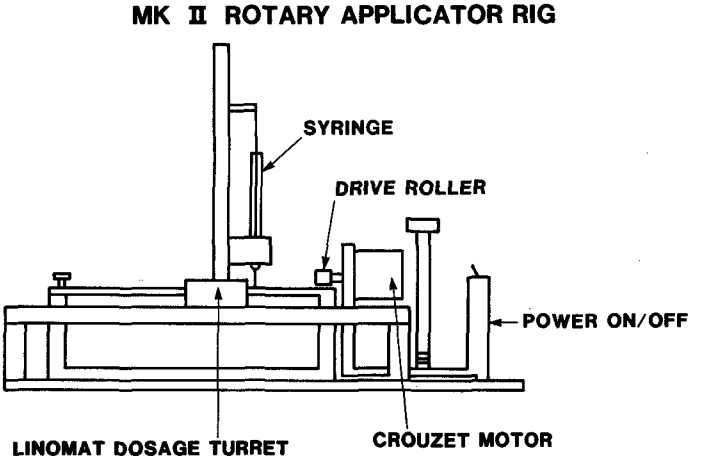


FIG. 2. Mark II rotary application rig.

were dissolved in redistilled chloroform and chloroform/methanol mixtures.

EXPERIMENTAL METHODS

Conditioning of Chromarods

The conditioning of each new set of Chromarods was carried out as recommended by the manufacturer. Chromarods were activated immediately prior to applying the sample by scanning in the Iatroscan TH-10. The Chromarods, when not in use, were stored in a glass humidity jar.

Sample Application

(a) Static Micro Capillary Method. One microliter of the sample solution was applied with a disposable capillary pipette to each of the 10 Chromarods held in the frame as described in the instruction manual.

(b) Static Agla Micrometer Syringe Method. One microliter of the sample solution was applied to each of the 10 Chromarods using the micrometer syringe. The polythene tip of the syringe was positioned so it just touched the surface of the Chromarod to allow the flow of sample solution onto the silica coating.

(c) Rotating Agla Micrometer Sample Method. Each Chromarod in turn was removed with forceps from the Chromarod frame and positioned in the rotary applicator rig (Fig. 1). With the aid of a magnifying glass, the polythene tip of the syringe was positioned to allow the transfer of the sample solution onto the Chromarod. The rotation of the Chromarods was started and a uniform application of the sample solution was made by rotating the Agla micrometer through 10 turns (10 revolutions = 1 μl) over a period of two min. This was equivalent to adding 0.1 μl per revolution of the Chromarod. The sample band width was observed continuously by viewing the Chromarod and the tip of the syringe through the magnifying glass. After the sample application was completed, the Chromarod was returned to its position in the development frame, followed by the transfer of the next Chromarod, and the procedure repeated for all 10 rods.

(d) Rotating Aerosol Sample Application Method. The specially designed Chromarod frame was placed in the sliding carriage which was an integral part of the MKII rig (Fig. 2). The frame was secured by two locating screws. The sample dosage turret was placed above the Chromarod frame by location onto two slide rails. Then the aerosol head was adjusted both for height (2 mm) above and alignment with the first Chromarod.

The motor drive was switched on and the rotating roller was lowered gently onto the exposed, uncoated end of the Chromarod by the tightening action of a clamp which distributed the pressure evenly over the sprung drive carriage. Once rotation of the Chromarod was obtained, the sample was dispensed by using the Linomat III dosage control.

Sample application was stopped automatically when it reached the preset value.

After sample application, the clamp of the drive roller unit was released and the drive roller automatically lifted away, allowing the Chromarod frame sliding carriage to be moved into the next position for application to the next Chromarod. This procedure was repeated until all 10 rods had sample applied. At the end of sample application, the Linomat dosage turret was removed and the Chromarod frame released from the rig.

Chromarod Development and Scanning

The Chromarods were dried in the drying chamber in a stream of nitrogen for five min at ambient temperature, transferred to the development chamber and developed. At the end of the development time (after the solvent front had reached the required height) the frame was removed and dried in a stream of nitrogen for five min. In the case of solvent systems containing ammonia solution, the frame and rods were heated at 110 C for a further five min.

The rods then were scanned in the Iatroscan, which was set up as described in the manufacturer's manual. For application methods (*a*) to (*c*), the rods were removed from their frame and transferred to the TH-10 analyzer. For method (*d*) the MKII specially built Chromarod frame was placed and positioned directly into the TH-10 analyzer by use of a locating screw.

Signal output was fed into the Hewlett Packard 3390A integrator, which plotted the chromatogram and gave peak areas and per cent normalized values of separated components for each Chromarod scanned.

RESULTS AND DISCUSSION

A mixture of ethoxylated anionic and ethoxylated nonionic commercial surfactants was separated to determine ratios of the two components. The separation of the mixture is shown in Figure 3.

Data from each run for each method of application were treated statistically to obtain a standard deviation (SD) for the set of 10 rods in the frame. Thus, the figures summarized in Table 1 are for interrod repeatability per run.

The data obtained by the micropipette

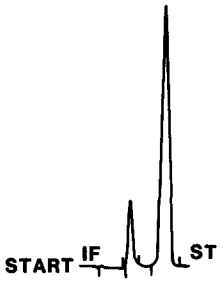
method of application gave poor repeatability within each set of 10 Chromarods, as shown by the SD figures in this table. The chromatogram shown in Figure 3 was a typical separation obtained using the micropipette method. This example illustrated the broad width of the peaks which also tend to tail.

A slight improvement was shown between this method and the Agla microsyringe non-rotating method. With both application methods there was a deterioration in repeatability with usage of a set of Chromarods, as shown by the data from the old and new sets.

The data obtained using the rotary application and Agla microsyringe showed a significant improvement in repeatability compared to the data obtained by sample application using the recommended method.

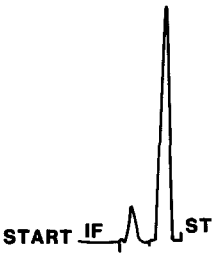
Rotary application gave symmetrical peaks of smaller peak width (Fig. 4). This probably was due to a more uniform distribution of sample charge around the Chromarod. With the recommended method of dropping the sample in one area, the ability of the components of low Rf to achieve uniform distribution around the rod while migrating only a small distance along the rod was greatly reduced. Another factor could be the possible variation of the silica coating along the Chromarod. The higher probability of spotting by the recommended method onto an area of silica which was not representative of the whole Chromarod also could lead to poor separation. It also should be noted that, with the rotating application method, both old and new Chromarods showed similar repeatability, holding out the possibility of extended rod life.

One of the limitations of the first three methods discussed was that the Chromarods had to be handled between the various operations of sample application, development and scanning. This increased the possibility of damage to the silica coating of the Chromarod. To overcome this problem the MKII applicator was designed to accept a Chromarod frame for sample application which also could be used for development and scanning in the Iatroscan without the need to manipulate the Chromarods. This unit also was designed so that the sample could be sprayed as an aerosol, using the dosage turret and modified syringe supplied with the Camag Linomat III.



ROTARY SAMPLE APPLICATION METHOD

FIG. 3. Sample application method recommended by Iatron Laboratories. Separation of anionic/nonionic surfactant mixture. Chromarod developed to 8 cm in ethyl acetate:ethanol:ammonia (65:22.5:12.5).



RECOMMENDED IATROSCAN SAMPLE APPLICATION METHOD

FIG. 4. Rotary sample application method. Sample and development as in Fig. 3.

TABLE 1

Range of SD for Each Run of 10 Chromarods

	Old Chromarods (B40)		New Chromarods (C71)	
	Nonionic	Anionic	Nonionic	Anionic
Traditional micropipette method (5 runs)	1.8-2.6	2.0-3.3	1.3-1.9	1.3-1.9
Agla microsyringe to non-rotating Chromarods (5 runs)	1.2-1.8	1.2-1.8	1.0-1.5	1.2-1.5
Agla microsyringe to rotating Chromarods (8 runs)	0.6-0.9	0.5-0.9	0.5-0.9	0.5-0.9
Aerosol application to rotating Chromarods (10 runs)			0.6-1.2	0.6-1.2

The modified applicator was used for lipid identification after extraction from sweet basil (*Ocimum basilicum*) tissue using a chloroform and methanol mixture. To avoid possible oxidation during concentration, the sample volume was increased to 20 μl of the dilute extract. This volume did not cause any problems for sample application, because rotation combined with the drying effect of the nitrogen stream from the aerosol applicator gave a uniform narrow band around the Chromarod. Therefore, by combining rotation of the Chromarod and application of the sample as a fine aerosol, concentration of dilute sample solutions was possible. This allowed greater confidence not only in the repeatability of rod to rod response, but also with the identification of component peaks. A typical chromatogram for a standard mixture is shown in Figure 5. This was a two-stage scan and development scheme to allow full identification of the multicomponent mixture.

Following the successful development of the MKII rig, it has been used for identification and quantification of surfactant materials supplied by a commercial manufacturer.

Figure 6 shows an example of chromatogram from the traditional and aerosol rotating method for a betaine surfactant mixture. The improvement of separation is recognized immediately. Another example is shown in Figure 7; this is an anionic/nonionic mixture of synthesized rosin acid surfactants. These chromatograms

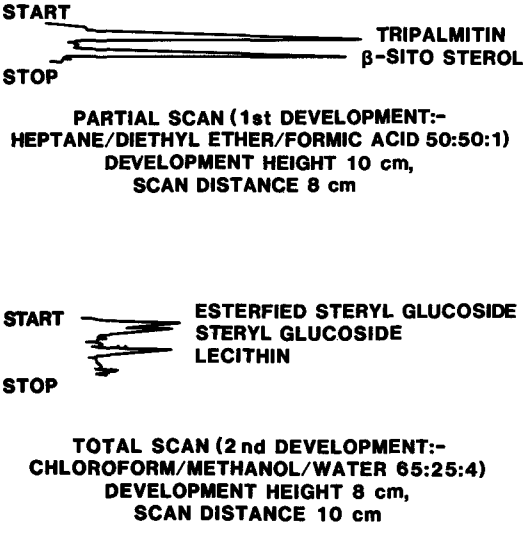


FIG. 5. Example of lipid analysis with multicomponent mixture applied by rotary method. Partial scan and redevelopment as shown.

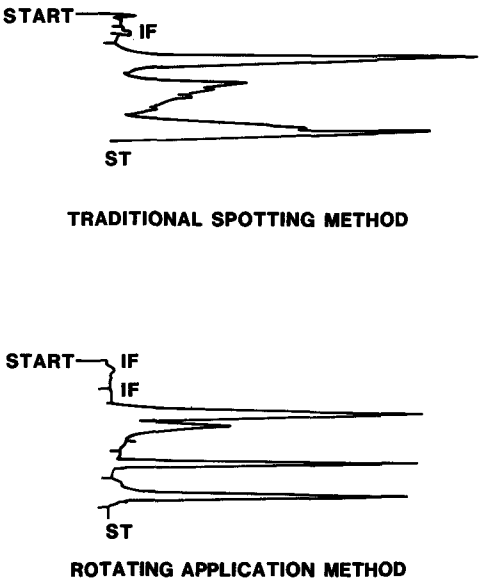


FIG. 6. Comparison of traditional and rotary sample application methods. Betaine surfactant mixture. Chromarods developed to 9 cm in chloroform:ethanol (2:1).

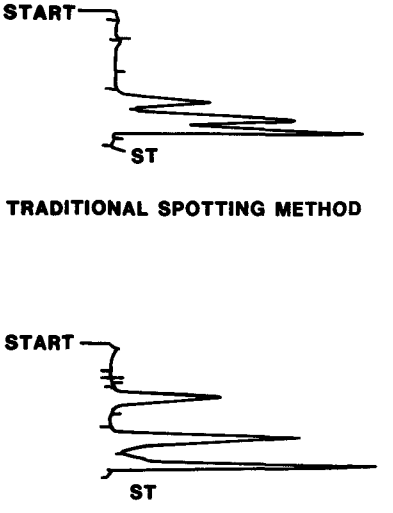


FIG. 7. Comparison of traditional and aerosol spotting methods with a surfactant mixture of rosin acid derivatives. Chromarods developed to 8 cm in chloroform:ethanol (9:1).

illustrate that component peaks are better separated and more symmetrical using the aerosol rotation method than with the traditional spotting technique. The interrod repeatability results for this method are included in Table 1. From these data it can be seen that this method gives an interrod repeatability range similar to the MKI rotation method, application method (*c*).

CONCLUSIONS

The use of the rotary method for the application and concentration of sample solution onto Chromarods gave improved interrod repeatability compared with the traditional method of sample application. Also, the higher resolution of the rotary method is invaluable for identification work based on retention data of known standards.

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Determination of the Phospholipid Composition of Trout Gill by Iatroscan TLC/FID: Effect of Thermal Acclimation

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ABSTRACT

The phospholipid composition of gill tissue from thermally acclimated rainbow trout, *Salmo gairdneri*, was determined by Iatroscan analysis following an initial development of the chromarods in a non-polar solvent to remove neutral lipids. Standard curves for all phospholipids, although linear through most of the concentration range tested (1-40 μg), extrapolated to negative intercepts on the ordinate, indicating a decline in sensitivity at low phospholipid levels. In addition, the concentration dependence of the Iatroscan response varied by nearly 6-fold among phospholipids. Of the major phospholipids, only lysophosphatidylcholine could not be quantitated accurately because of the presence of an interfering peak. Quantitation by Iatroscan yielded results which, in general, agreed well (within 5%) with results obtained by an independent phosphate analysis. Only in the case of phosphatidylinositol (PI) did the two analytical methods differ significantly; proportions of PI were 55% higher when determined by Iatroscan as opposed to phosphate analysis. Gill tissue from 5 C-acclimated trout possessed higher proportions of phosphatidylethanolamine than tissue from 20 C-acclimated trout. The Iatroscan provided a rapid and reliable means of quantitating the proportions of all the major phospholipids of trout gill, although there are some limitations to the general applicability of the technique.

Lipids 20:516-520, 1985.

INTRODUCTION

This study was undertaken to evaluate the use of the Iatroscan TLC/FID system in separating and quantitating the phospholipid class composition of total lipid extracts from rainbow trout gill. An initial development of the Chromarods in a non-polar solvent was employed to separate neutral lipids from the phospholipid fraction. A second development in a more polar solvent was used to resolve individual phosphatides. Although double-development procedures for the analysis of phospholipids in total lipid extracts have been described previously (1,2), quantitative data are limited, and the results of Iatroscan analyses have not been compared with those of independent analytical methods. In addition, the applicability of this technique to diverse tissue types remains to be established.

In the present study, the effect of acclimation temperature upon the phospholipid composition of trout gill is determined by Iatroscan analysis. In addition, these results are compared with quantitation achieved by phosphate analysis following the isolation of individual phospholipids by two-dimensional thin layer chromatography.

MATERIALS AND METHODS

Animals

Rainbow trout, *Salmo gairdneri*, ranging in body weight from 100-250 grams (mean 217 ± 42, n=10) were obtained from the Alchesay National Fish Hatchery in Whiteriver, Arizona and acclimated in the laboratory to water temperatures of either 5 C or 20 C on a 12-hr photoperiod for at least one mo prior to experimentation. All fish were fed Glencoe Mills trout food (the composition of which has been reported previously [3]) once daily.

Chemicals

Organic solvents were of analytical reagent grade, washed free of impurities if necessary (4) and freshly redistilled prior to use.

Phospholipid standards were obtained from Supelco, Inc. (Bellefonte, Pennsylvania). Bio-Sil A was from Bio-Rad Laboratories, Richmond, California. All other chemicals were analytical reagent grade; water was deionized and glass-distilled.

Lipid Extraction

Fish were killed by a blow to the head and drained of blood by cardiac puncture. Gill tissue was excised in ice-cold 0.9% NaCl prior to being blotted and weighed; only the gill filaments were extracted. A total lipid extract of the gill tissue was prepared by homogenization in 20 volumes of $CHCl_3$/MeOH (2:1, v/v). The lipid extract subsequently was washed with 0.88% KCL as described by Folch et al. (5). The organic phase was dried by codistillation with benzene and stored at -20 C in a defined volume of C/M (2:1).

Phospholipid Composition—Phosphate Analysis

Gill phospholipids were isolated from neutral and glycolipids by column chromatography on Bio-Sil A as described previously (4). Individual phosphatides were resolved by two-dimensional TLC ($CHCl_3$/MeOH/ammonia, 65:35:5 in the first dimension; $CHCl_3$/MeOH/HOAc/H_2O, 25:15:4:2 in the second dimension) on plates of silica gel H containing 0.77% magnesium acetate (6). Following visualization with iodine vapor, phospholipid spots were scraped, neutralized, digested with $HClO_4$ and assayed for phosphate as described by Rouser et al. (7).

Phospholipid Composition—Iatroscan TLC/FID

Chromarods-SII, routinely stored in 6M HNO_3, were run through five washes of distilled water (5 min each) prior to being heated at 120 C for one hr. Rods were then scanned and spotted immediately with 1 μl (in 5 0.2 μl applications) of total lipid extract (total application, 100 μgrams lipid). The development protocol consisted of the following steps: (i) a 15-min incubation at 52% relative humidity over a saturated solution of $Na_2Cr_2O_7$; (ii) equilibration with vapors of the first developing solvent (hexane/ether/formate, 80:20:2)—15 min; (iii) development in the first solvent—30 min; (iv) solvent drying—15 min at room temperature; (v) scan for neutral lipids—the last 1 cm of the rod containing the origin (and phospholipids which did not migrate in the first solvent system) was not scanned; (vi) incubation at 52% relative humidity—15 min; (vii) equilibration with vapors of the second solvent system ($CHCl_3$/MeOH/H_2O, 80:35:3 [1])—15 min; (viii) development in the second solvent—40 min; and finally, following oven drying at 120 C for 5 min, the entire length of the Chromarod was scanned for phospholipids. The scanning rate was 32 sec/rod, the hydrogen pressure was 0.75 kg/cm^2, the air flow 2 l/min and the chart speed 10 sec/in. (the Iatroscan was a model TH-10, mk. III). Peak areas were quantitated by cutting and weighing. Weight percentage values were computed after converting peak weights to grams of phospholipid employing the linear regression equations described in Table 2.

Statistical Methods

Tests for statistical significance were made following arc sine transformation of the percentage data employing an analysis of variance for planned comparisons (8).

RESULTS

As illustrated in Figure 1, preliminary development of the Chromarods in hexane/ether/formate resulted in the elution of two major neutral lipid peaks. No attempt was made to identify these neutral lipid components. Subsequent development of the Chromarod in $CHCl_3$/MeOH/H_2O resulted in a 'phospholipid scan' reproducibly comprised of seven distinct peaks (Fig. 1). Because solvent fronts were difficult to visualize, Rf values were calculated relative to phosphatidylethanolamine (PE—peak 2). Peaks 1-6 were identified as bona fide phospholipids by comigration with authentic phospholipid standards (Table 1). In contrast, peak 7 did not migrate with authentic lysophosphatidylcholine (LPC) and comprised a much larger percentage of the area scan (5-9%) than the percentage composition of LPC (1.52 ± 0.15) determined by phosphate analysis. For these reasons, peak 7 is believed to be either an unidentified lipid or an artifact resulting from the initial neutral lipid scan (the position of this peak corresponds approximately to that point on the Chromarod where the first scan stopped). The presence of peak 7 in these samples made the quantitation of LPC impossible, because it is larger than and obscures the true LPC peak.

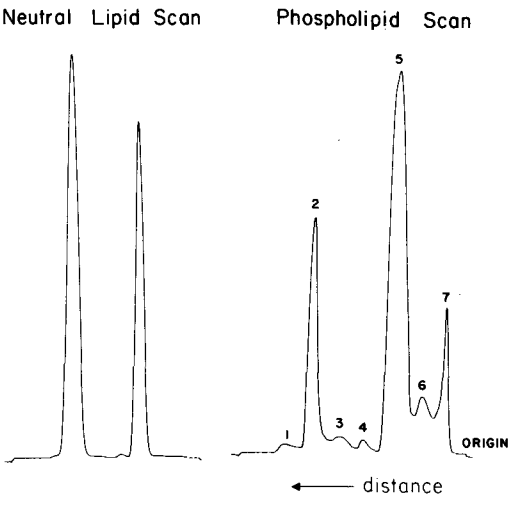

FIG. 1. Trout gill lipids separated on Chromarods-SII. The neutral lipid scan was obtained following development in hexane/ether/formate (80:20:2); the final 1 cm of the rod (containing the origin) was not scanned. The phospholipid scan was determined following a second development of the rod in $CHCl_3$/MeOH/H_2O (80:35:3). Identified components: 1, cardiolipin; 2, phosphatidylethanolamine; 3, phosphatidylinositol; 4, phosphatidylserine; 5, phosphatidylcholine; 6, sphingomyelin; 7, undetermined (see text).

Standard curves for authentic phospholipid standards are illustrated in Figure 2 and conform to linearity throughout most of the concentration range tested. However, the y-intercepts

TABLE 1

Summary of Relative Rf's of Phospholipid Standards and Trout Gill Phospholipids

	Standards			Gill phospholipids		
Phospholipid	CM traveled	Rf*	N	Peak #	Rf*	N
Cardiolipin	6.49 ± 0.36	1.06	11	1	1.12 ± 0.02	30
P-ethanolamine	6.10 ± 0.31	1.00	13	2	1.00	–
P-inositol	5.24 ± 0.24	0.86	10	3	0.83 ± 0.02	30
P-serine	3.74 ± 0.26	0.61	8	4	0.64 ± 0.02	30
P-choline	2.77 ± 0.25	0.45	15	5	0.45 ± 0.02	30
Sphingomyelin	1.91 ± 0.16	0.31	14	6	0.31 ± 0.02	30
Lyso-P-choline	1.53 ± 0.15	0.25	12			
				7	0.15 ± 0.01	30

Mean ± SD.
*Relative to PE.

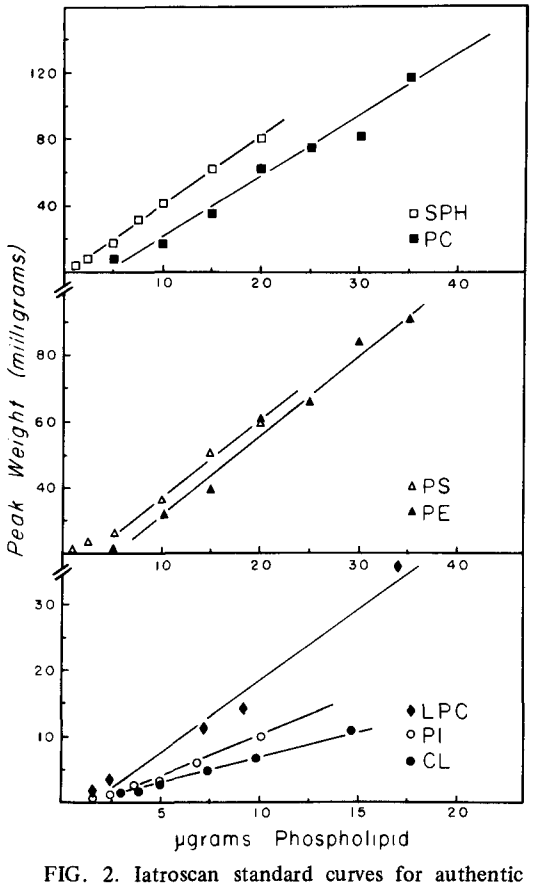

FIG. 2. Iatroscan standard curves for authentic phospholipid standards. The lines drawn through the data points are least square linear regression lines (see Table 2).

of the least square regression lines (Table 2) are in all cases negative, indicating deviations from linearity at low phospholipid concentrations. The extent of this deviation varied considerably depending on the phospholipid and was most

TABLE 2

Summary of Least Square Linear Regression Parameters for Phospholipid Standard Curves Determined by Iatroscan Analysis

Phospholipid	Y-Intercept	Slope (mg/μg)	R^2
Cardiolipin	- 0.62	0.74	0.9999
P-ethanolamine	-13.12	3.49	0.9956
P-inositol	- 1.97	1.21	0.9996
P-serine	- 3.26	2.15	0.9897
P-choline	-17.01	3.76	0.9941
Lyso-P-choline	- 0.59	3.04	0.9823

marked for phosphatidylcholine (PC) and PE. Furthermore, the concentration dependence of the Iatroscan response (as incicated by the slopes of the regression lines–Table 2) varied nearly 6-fold among the phospholipids tested. The concentration of the lipid extract applied to the Chromarods was adjusted so that the quantities of all phospholipids fell within the linear range of the standard curves.

Figure 3 compares the phospholipid composition of gill tissue from 20 C-acclimated trout determined by Iatroscan analysis (employing the linear regression equations reported in Table 2) and by phosphate analysis of the same lipid samples following 2-dimensional TLC. Peak 7 was excluded from the Iatroscan analysis for the reasons discussed above. Although LPC is present in trout gill, it comprised only 1.5% of the total lipid phosphate. Thus, the exclusion of peak 7 from the analysis does not result in a significant error. Furthermore, the phosphate values shown for cardiolipin are actually the sum of cardiolipin and phosphatidic acid because these two phosphatides are not resolved by Iatroscan analysis (phosphatidic acid was a very minor constituent of trout gill, averaging 0.67 ± 0.17% of the total lipid phosphate). In general, the agreement between the two analytical

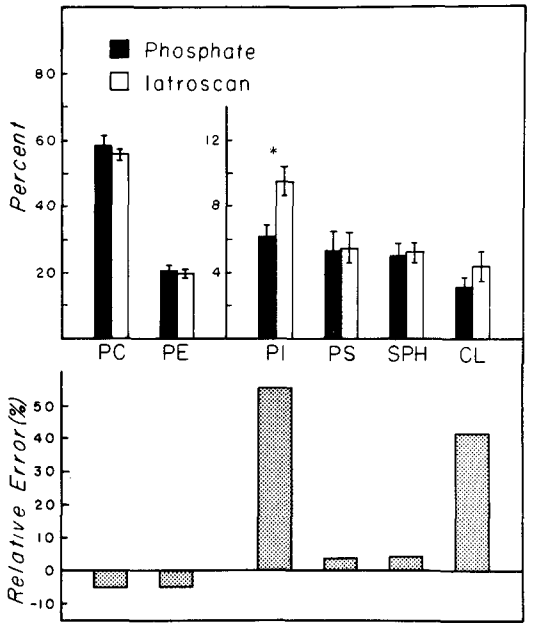

FIG. 3. Relative phospholipid compositions of gill tissue from 20 C-acclimated trout determined by Iatroscan analysis and by phosphate determination following 2-dimensional TLC. The phosphate values shown for cardiolipin are the sum of cardiolipin and phosphatidic acid. The percentages based on phosphate analysis include the contribution of LPC; those based on the Iatroscan analysis do not (see text). Presented as mean ± SD. Symbols and abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SPH, sphingomyelin; CL, cardiolipin; *, $p < 0.05$.

TABLE 3

Phospholipid Compositions of Gill Tissue from 5 C- and 20 C-Acclimated Rainbow Trout Determined by Iatroscan TLC/FID

Phospholipid	Weight percentages (n = 5)	
	5 C-acclimated	20 C-acclimated
Cardiolipin	6.20 ± 0.49	4.36 ± 0.41*
P-ethanolamine	22.86 ± 0.61	19.86 ± 0.33*
P-inositol	8.32 ± 0.25	6.51 ± 0.40
P-serine	4.14 ± 0.20	5.47 ± 0.44
P-choline	53.56 ± 1.19	55.55 ± 0.79
Sphingomyelin	4.94 ± 0.09	5.24 ± 0.25
PC/PE	2.24 ± 0.21	2.80 ± 0.03*
Total recovery	100.00 ± 2.83	99.99 ± 2.62

Mean ± SEM.
*$P < 0.05$.

methods is remarkable. Only in the case of PI did the Iatroscan analysis yield significantly different values than the phosphate analysis; for all other phospholipids, with the exception of cardiolipin, the two analyses agreed to within 5% of one another.

The phospholipid compositions of gill tissue from 20 C- and 5 C-acclimated trout determined by Iatroscan analysis are compared in Table 3. Gill tissue of cold-acclimated trout possessed significantly higher levels of cardiolipin and PE and a significantly lower ratio of PC/PE than tissue from warm-acclimated trout.

DISCUSSION

The results presented demonstrate that the proportions of all major phospholipids present in total lipid extracts of trout gill may be determined by Iatroscan TLC/FID without first isolating a purified phospholipid fraction. There are, however, some potential limitations to the general applicability of this technique which deserve comment. First, it was not possible to accurately quantitate the amounts of LPC due to the presence of an interfering peak. In the present case, the exclusion of LPC from the Iatroscan analysis did not result in a significant error, for phosphate analysis indicated that LPC is only a minor constituent of gill phospholipids. Second, the linear range of the standard curve varies with the phospholipid. Consequently, the amount of the sample spotted needs to be adjusted so that each component in the mixture falls within the linear range of its standard curve. And third, the response of the Iatroscan varies significantly among phospholipids, necessitating the construction of a separate standard curve for each component present in a mixture.

In spite of these limitations, the results of the Iatroscan analysis agreed favorably with quantitation achieved by phosphate analysis. The relative error for the two methods was nearly identical, and only in the case of a single phospholipid (PI) did the two methods give significantly different results. In addition, the significantly higher proportions of PE and the lower ratio of PC/PE determined for gill extracts from cold- compared to warm-acclimated trout are consistent with the effects of acclimation temperature upon the phospholipid composition of membranes from a variety of poikilotherms (9).

In summary, the determination of the phospholipid composition of trout gill by Iatroscan-TLC/FID yielded results which were in generally good agreement with the results of conventional phosphate analysis. There are some limitations

to the general applicability of the technique, but an appreciation for the factors that influence the analysis can eliminate significant errors.

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Calibration of the Iatroscan-Chromarod System for Marine Lipid Class Analyses

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ABSTRACT

A two-step development procedure with partial scanning after the first development resolves seawater lipids into seven classes. The low concentration in seawater of some of these classes necessitates calibration close to the detection limit of the flame ionization detector (FID). From 0.2 to 5.0 μg the FID response usually is curvilinear, necessitating multilevel calibration. Interrod precision was poor for most of this range, and this prompted an investigation of factors affecting FID responses.

Peak areas were found to depend on the distribution of lipid material on the Chromarod. Also investigated were the effects of temperature, humidity, acid cleaning and double developments. All of these factors had some effect on R_f values, on absolute FID responses and on reproducibility, but they were not the major cause of variability in the Iatroscan-Chromarod system. Similarly, double developments improve peak shape and response, but result in only a small increase in precision for some compounds. The differences among the FID responses obtained from 10 rods within a set imply that the normalization of FID responses to that of an internal standard, or the use of intrarod rather than interrod data, should result in an increase in reproducibility. Neither of these approaches was found to improve the precision for all lipid classes. An understanding of both interrod and intrarod variability, and the use of calibration curves, is essential for accurate determination of the lipid classes of 1 liter of seawater.

Lipids 20:521-530, 1985.

INTRODUCTION

In contemporary oceanography, there are few other techniques currently available which can compare with TLC/FID for the number of marine organic classes that can be measured, the number of samples that can be processed, or for the simplicity and price of equipment required.

The Iatroscan-Chromarod system would appear to be well suited for an examination of marine organic classes in an oceanographic context. Thin-layer separations on the reusable Chromarods are both excellent and versatile (1, 2), the Iatroscan flame ionization detector (FID) system is robust enough to function well at sea (3), and the scanning time is very rapid: a chromatogram is obtained in less than a minute. However, the relationship between FID response and compound load is not straightforward (3-5), and it has been suggested that the Iatroscan has limited applicability in the quantitative analysis of lipids (1,4).

The present study is a detailed examination of the magnitude and precision of FID responses of lipids used as representatives of marine classes separated on Chromarods.

MATERIALS AND METHODS

Apparatus and Operating Conditions

All separations were performed on silica-coated Chromarods-SII. Shipboard FID scans (Fig. 1) were performed on an Iatroscan (Iatron Laboratories, Tokyo) Mark II analyzer which had the analogue and integral outputs connected to a two-pen Fisher Recordall (3). Land-based studies on FID response and precision were performed on an Iatroscan Mark III connected, via the analogue output, to a Spectra-Physics SP4200 computing integrator. Both Iatroscans were fitted with a push-button switch (normally closed) placed in a series with the microswitch (S_1) used to stop scans. With this arrangement any Chromarod FID scan could be interrupted at any time. The Iatroscans were operated with a hydrogen flow of 160 ml/min, a scan speed of 3.1 mm/sec and an air flow of 2000 ml/min.

Standards (Table 1) were dissolved together in chloroform or (in some cases) singly in isooctane. Standard solutions were spotted onto Chromarods with disposable 1 μl pipettes (Drummond Scientific Co., Broomall, Pennsylvania). All solvents used were 'distilled in glass' grade or better.

Experimental Procedure

Lipids were applied to flame-activated Chromarods and focused twice to the origin in acetone (3). The rods were deactivated for 10 min over a saturated solution of NaCl, and then equilibrated with the vapor of the first developing solvent system for 5 min before development. The lipids were separated in a stepwise sequence

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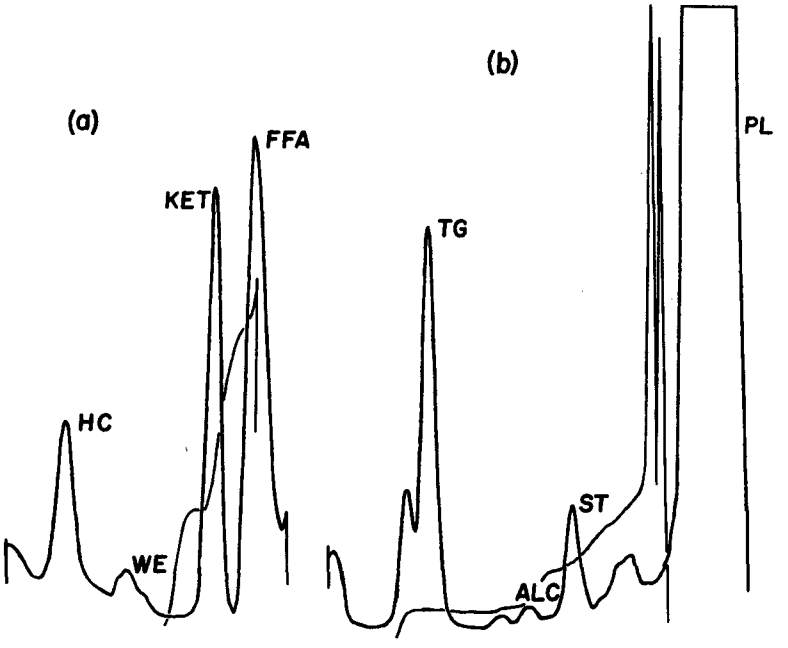

FIG. 1. Shipboard analysis of dissolved lipids in Scotian Shelf waters; abbreviations explained in Table 1. (a) 45-min development in hexane/diethyl ether/formic acid (98:2:0.2); partial scan. (b) Second development on the same rod: 45 min in hexane/diethyl ether/formic acid (80:20:0.2); full scan. Developing direction is from right to left, scanning direction is from left to right.

TABLE 1

Seawater Lipid Classes and Standards Used for their Identification and Calibration in the Iatroscan

Class	Abbreviation	Standards and Suppliers
Aliphatic hydrocarbon	HC	Nonadecane (Polyscience)
Wax and sterol esters	WE	Hexadecyl palmitate (Analabs)
Ethyl ketone	KET	Hexadecan-3-one[a] (K & K Labs)
Free fatty acid	FFA	Palmitic acid (Supelco)
Triglyceride	TG	Tripalmitin (Supelco)
Free alcohol	ALC	Hexadecan-1-ol (Polyscience)
Free sterol	ST	Cholesterol (Supelco)
Polar lipid	PL	Dihexadecanoyl lecithin (Supelco)

[a]Internal standard.

(2). After each day of use the rods were acid cleaned (5) and stored over a saturated solution of NaCl. Shipboard seawater analyses (Fig. 1) were performed on dichloromethane extracts of 1 liter aliquots of filtered water (3).

RESULTS AND DISCUSSION

Separations

A two-step development strategy was used to perform shipboard analyses of 'dissolved' lipid classes in filtered seawater (Fig. 1). The first solvent system resolves classes in the polarity range HC to FFA [Fig. 1(a): abbreviations are explained in Table 1]. The rod scan is stopped, using the push-button switch, at the lowest point on the tail of the FFA peak, and the remaining unpyrolyzed classes are then developed with a more polar solvent system [Fig. 1(b)]. This strategy resolves seven seawater lipid classes which have been identified by R_f values and by cospotting (Table 1).

Calibration Curves

For practical reasons it is necessary to reduce seawater sample size as much as possible when

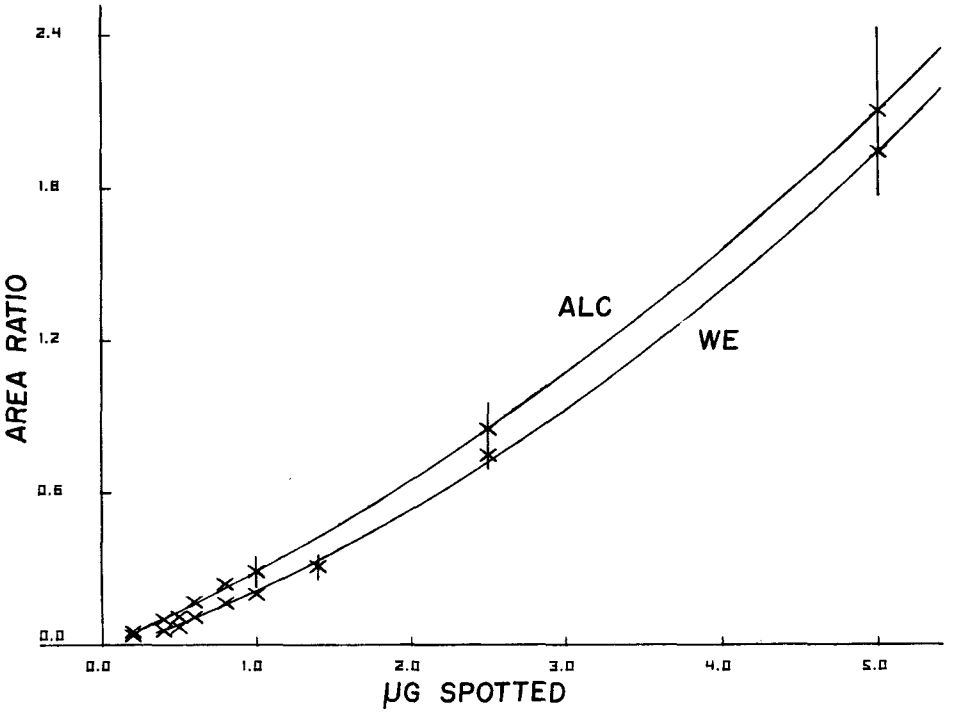

FIG. 2. Calibration curves. Computer-drawn quadratic regressions (Table 2) through response data from hexadecanol and hexadecyl palmitate taken in ratio to 3 μg of hexadecanone; error bars extend 1 standard deviation from the mean.

performing shipboard analyses. This means that the FID detector in the Iatroscan has to be operated quite near its detection limit for most lipid classes in most seawater samples, and detailed low-load calibration curves are necessary for the minor seawater lipid classes.

To investigate the linearity and precision of the FID response at low μg-loads, a single set of 10 Chromarods was used. On average, eight different loads were applied to the Chromarods, and about half of these loads were below 1 μg (Figs. 2 and 3). Coefficients of determination (r^2) were calculated for five different regression models (Table 2). In order to obtain an estimate of the precision of the analyses over this range, the coefficient of variation (CV) was calculated at each level, and it is the mean over the range 0.2 to 5.0 that is given in Table 2. The CV is the standard deviation expressed as a percentage of the mean, and in Table 2 six analyses were made, on average, with each μg-load.

Calibration curves in the range 0.2 to 5.0 μg are distinctly curvilinear (Figs. 2 and 3), and are best described using quadratic equations (Table 2). There is, however, only a small difference in the r^2 values for quadratic and linear regressions, but the latter pass through the x axis between 0.2 and 0.5 μg and are thus unsuitable for calibration at low loads.

A positive intercept on the load axis from a linear regression (6) will automatically result in an increase in response per μg with increasing load (4), because the equation describing the response contains a negative term. Any positive curvature [Table 2, (7-9)] will further aggravate this situation. Thus, single-level calibrations (10,11) should be used only when similar loads of samples and standards are scanned on Chromarods. When a range of lipid loads is to be analyzed, a multilevel calibration (Figs. 2 and 3) will produce more accurate results.

There also is little difference between the correlations for quadratic and power law regressions (Table 2). Power law calibrations have been applied to lipids scanned on boric acid impregnated Chromarods (12). The good fit obtained with power curves suggests similarities with nonlinear FID responses obtained from lipids after liquid chromatographic separations (13) or else after gas chromatographic separations (14).

By taking logarithms of both sides of a power

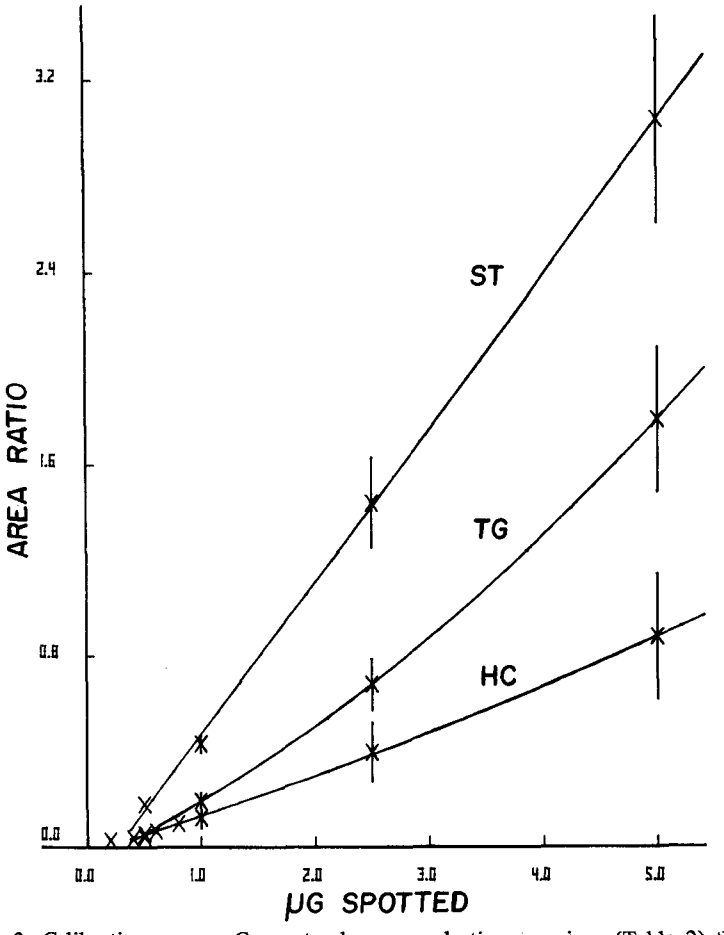

FIG. 3. Calibration curves. Computer-drawn quadratic regressions (Table 2) through response data normalized to responses from 3 μg of hexadecanone; error bars extend 1 standard deviation from the mean.

TABLE 2

Iatroscan FID Responses: Precision and Regressions at Low Loads (0.2 - 5.0 μg)

		r^2 Values					Quadratic constants		
Class	Mean CV	ae^{bx}	ax^b	a+b1nx	a+bx	$a+bx+cx^2$	a	b	c
HC	23	.874	.975	.805	.977	.999	.023	.15	.007
WE	17	.865	.984	.725	.985	.999	.030	.21	.038
TG	16	.883	.996	.864	.993	1.000	.067	.23	.029
ALC	19	.866	.995	.789	.993	1.000	.101	.27	.031
ST	11	.894	.998	.909	1.000	1.000	.164	.63	.002

law equation [1], the equation for a straight line is obtained [2]:

$$y = ax^b \quad [1]$$

$$\log y = \log a + b(\log x) \quad [2]$$

With Iatroscan data y, the dependent variable, is the measured peak area; x, the independent variable, is the load applied to the Chromarod; and log a, and b are constants; the y-intercept and slope of the line in equation [2]. If b = 1, the FID can be said to respond linearly (14).

Linear calibration curves have been obtained previously from Iatroscan data plotted on full

logarithmic graph paper (15,16). This suggests further support for the applicability of a general power law model to describe Iatroscan FID response.

Reproducibility

In the range 0.2 to 5.0 μg there is a general gradation in the standard deviation of the mean FID responses (Figs. 2 and 3). As noted previously (7), lower loads have lower standard deviations as do compounds with lower FID responses. The trend for the CV, however, is usually the opposite (15,17). Thus, the mean CV over a range that includes several data points at low loads is usually high (Table 2). Standard deviations significantly higher than 10% of the mean have been reported previously for low μg-loads (3,7,15,17).

The increase in standard deviation with increasing load suggests that simple linear regressions through the logarithms of the calibration data would be more accurate than nonlinear regressions through the untransformed data. This would not be the case if weighting factors were used with the nonlinear regressions. This argument has been applied to TLC-densitometry calibration data, which also is nonlinear (18). However, it should be noted that although the use of log-transformed data produces linear Iatroscan calibration curves (15,16), it does not obviate the necessity of performing multilevel calibrations.

The generally poor CV obtained for low loads with most of the representatives of the classes that usually are found as minor components in the lipids of seawater (Fig. 1; Table 2) prompted some detailed investigations into factors affecting FID responses.

Modification of FID Responses

A rapid method for collecting a large amount of FID data is to spot the same compound at various places on each of a set of 10 rods and to scan these rods without having developed the spots (4,5). Using this approach, a relationship between integrator peaks and solvents used in standard solutions was established. Hexadecanone spotted in 1 μl of isooctane gives tall, thin peaks whose area depends on the orientation of the Chromarod with respect to the FID burner. With rod orientation reversed, so that the side of the rod that was spotted passes nearest the top of the FID burner, the FID response was significantly higher. Rod orientation had no effect on hexadecanone spotted in chloroform. However, the peaks produced using chloroform as a spotting solvent were often broad and frequently were split near the top. The same situation occurs with tripalmitin spotted in chloroform/methanol 2:1 (5). The differences in peak shape resulting from spotting in the isooctane solution or the chloroform-based solutions are likely to be related to differences in solvent polarity and the ability of the solvent to move the material away from the point of application. With hexadecanone spotted in isooctane the lipid material must remain not only at the point of application in terms of displacement along the rod, but also in terms of displacement around the rod. Since there rarely is any sign of a peak on reburning the rod in any orientation, it appears that the distribution of material influences the proportion of ions registered by the integrator after being detected by the FID.

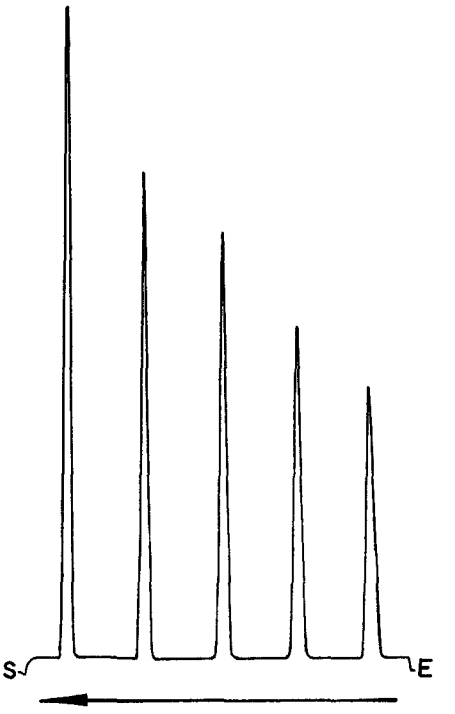

FIG. 4. Hexadecanone spotted in 1 μl of isooctane at 5 separate places on a Chromarod. The rod was developed, from right to left in the figure, for 40 min in 100% hexane; S and E denote the start and end of the FID scan.

It was anticipated that more regular peak shapes would be obtained from a rod that has been spotted at several places if the lipid material was developed. In order to avoid running separate peaks together it is necessary to use a solvent in which the lipid material is only slightly mobile (Fig. 4). This development almost invariably eliminates the appearance of split peaks in chromatograms; however, it causes a gradation in peak shapes along the rod (Fig. 4).

The lipid material which has spent the longest period of time in the developing solvent has the broadest-based peak. This corresponds to the familiar band spreading found in other chromatographic techniques. However, what is unexpected about these peaks is that there is a concomitant gradation in peak area along the rod: the broadest-based peaks have a lower area than the rest. This applies to both hexadecanone and tripalmitin (5). These observations, coupled with those from undeveloped spots, suggest that there is a relationship between the distribution of material on the Chromarod, the shape of the peaks produced on scanning, and the final area that is integrated.

If hexadecanone and tripalmitin are spotted together and developed in hexane/diethyl ether (80:20) they migrate to the same place on the Chromarod. The total peak area, however, is significantly larger than the sum of the areas of each compound when developed singly to the same place. This suggests that the increase in peak area per μg with increasing load (Figs. 2 and 3) does not depend directly on the nature of the material being pyrolyzed. It seems likely that the FID responds nonlinearly to the number of ions produced per unit area of Chromarod; or, for a given scan speed, it responds curvilinearly to the flow of ions per unit time. This argument may help explain why some compounds have a higher response at higher scan speeds (4). It also could help explain the differences in peak area observed with different band-widths of the same amount of material (Fig. 4). The FID passes through a complete range of responses as a band of material is burnt on a Chromarod. With a narrow band the FID will respond at a proportionally higher level on average than with a broad band, even if identical amounts of material are burnt in both cases. Thus the narrower band automatically will give a larger peak area.

Factors Affecting Silica Gel-Lipid Interactions

The indication of an apparent association between peak area and the distribution of lipid material on the silica suggested that an improvement in the reproducibility of the migration of a compound could lead to an improvement in the precision of the quantification of that compound.

It has long been known that the reproducibility of R_f values on TLC plates can be improved by controlling the relative humidity of the atmosphere in contact with the plate prior to development (19). A 10-min exposure of Chromarods to a constant relative humidity (between 30 and 75%, depending on the saturated salt solution used) does improve the reproducibility of R_f values when there are large

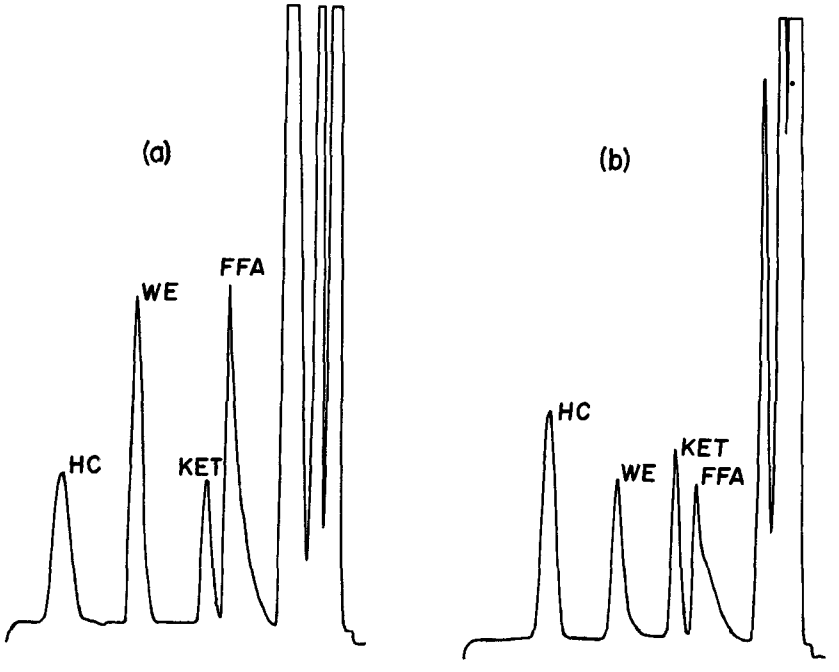

FIG. 5. Complete scans after 40-min developments, at (a) 24 C and (b) 3 C, of the same standard solution on the same rod in hexane/diethyl ether/formic acid (97.9:2:0.1).

changes in the relative humidity in the laboratory. However, it was found that on a run to run basis the improvement in the reproducibility of R_f values and the improvement in the precision of quantification were not statistically significant.

Similar conclusions can be drawn from other factors which might be considered to affect R_f values or FID responses. Developments performed at very different temperatures result in different R_f values and different FID responses (Fig. 5); however, the use of a constant temperature bath to maintain the developing temperature within a 0.3 C range did not result in a significant increase in precision.

After several developments on a set of Chromarods the R_f values and FID responses differ from those obtained from rods that have just been acid cleaned. However, it was again not possible to demonstrate an improvement in the standard deviation of the mean when rods were used for a maximum of only two development sequences before being acid cleaned.

These observations imply that, although a strict control of factors which can affect the migration of lipids does appear to improve the reproducibility of FID responses, the factors that were investigated are not the main cause of rod to rod or run to run variability in Iatroscan data.

Double Developments

The effect of developing solvents on recorder peak shapes (Fig. 4) suggested an investigation into the effect of double developments on FID responses and their reproducibility. A direct parallel to the gradation of peak shapes and areas (Fig. 4) can be found in chromatograms of neutral lipid standards [Figs. 6(a) and 7(a)]. HC, the most mobile class, gives the broadest-based peaks [Fig. 6(a)] and also the lowest FID response (Fig. 3). By producing a narrower peak it should be possible to increase the peak area [Fig. 4, (5)]. For HC this can be achieved by performing a second development, in the same solvent system, for a shorter length of time. This procedure does, on average, increase the peak area [Fig. 6(b)]. A double development of the TG peak produces similar results [Fig. 7(b)]. This time, however, two different solvent systems were used. The three remaining neutral lipid standards were moved from the point of application with a 40-min development in hexane/diethyl ether (80:20). After drying, the rods were redeveloped in hexane/diethyl ether/formic acid (98:2:0.1) for 30 min and then subjected to a partial scan to just after the ST peak [Fig. 7(b)].

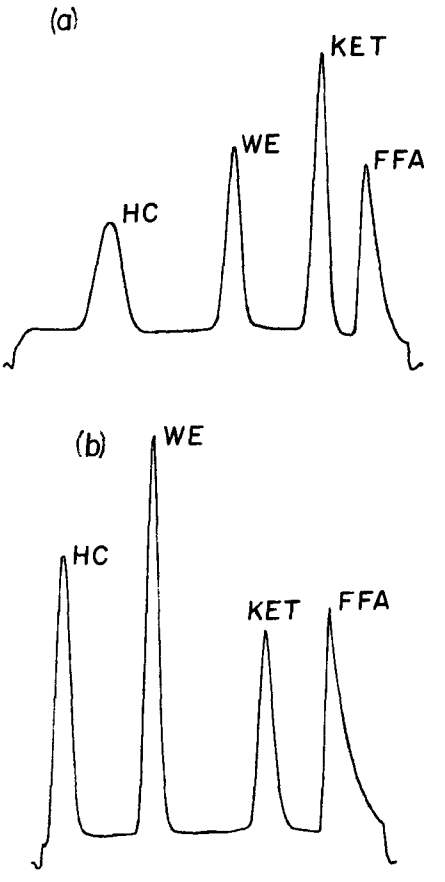

FIG. 6. Partial scans of 3 μg of each compound on the same rod; (a) after a 40-min development in hexane/diethyl ether/formic acid (98:2:0.1), and (b) after a double development (40 followed by 30 min) in the same solvent system.

In most neutral lipid separations, PL remains at the point of application and gives a comparatively large peak area considering its high proportion of oxygen [Fig. 7(a)]. Since PL is immobile in the solvent systems used it would seem that this is analogous to the situation described above for undeveloped rods, and thus the shape of the PL peak is almost entirely dependent on the polarity of the spotting solvent. If this analogy is correct, development of PL away from the point of application should reduce the peak area. This is indeed the case [Fig. 7(b)]. The phospholipid standard was moved from the origin with two 5-min developments in chloroform/methanol/water (50:40:10). After drying, the rods were subjected to a full scan. The chart speed on the computing integrator was routinely set at a slower rate for this scan and for reburns done immediately after it (Fig. 7), in order to save paper.

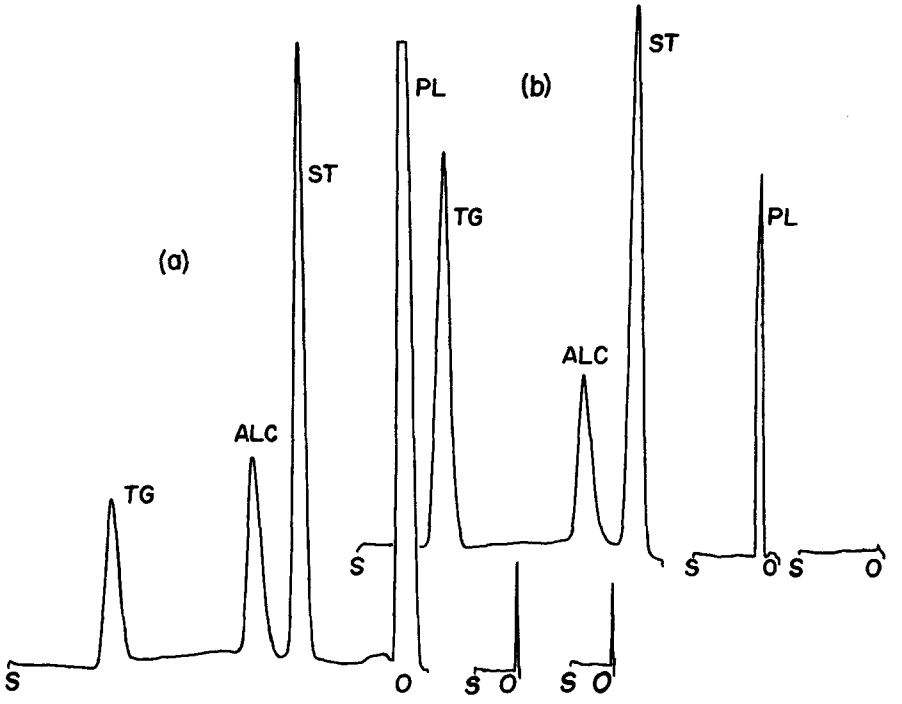

FIG. 7. Developments, scans and reburns after the partial scans in Fig. 6; attenuation is the same for all chromatograms, chart speed is slower for second scans and for reburns; 0 is the point of application, S denotes the starting point of a scan. (a) Complete scan after development for 40 min in hexane/diethyl ether (80:20), followed by two reburns (bottom center) at a slower chart speed. (b) Partial scan of neutral lipids after a double development in hexane-based solvent systems, followed by a complete scan at a slower chart speed after a double development in a chloroform-based solvent system, followed by a reburn (far right).

A further difference between developed and undeveloped PL peaks is the response on reburning the Chromarod (Fig. 7). Developed PL peaks rarely show as much as 1% of the original peak remaining on a reburn [Fig. 7(b)]. A reburn of undeveloped peaks, however, often results in a residual peak which is larger than 1% of the original area [Fig. 7(a)]. It is unlikely that a major proportion of this residual peak consists of the lecithin that was spotted. This material is immobile in phospholipid solvents, and the peak is not reduced by a further 95 to 99% on reburning [third scan of the rod at the point of application: Fig. 7(a)]. In fact, it takes more than 10 scans or else strong acid cleaning to reduce the peak to an undetectable level. The fact that development of the PL peak in a very polar phospholipid solvent alleviated the problem of peaks appearing on recombustion [Fig. 7(b)] implies that residue peaks on Chromarods can be an artifact of the distribution of material on the Chromarod. Some of these observations are similar to those suggested to be the result of the inclusion of phytin in polar lipid fractions scanned on Chromarods (20).

The ease with which FID responses can be altered by different lengths of exposure to solvent systems (Figs. 4, 6 and 7) implies that it would be difficult to make comparisons between FID responses in the literature (4) unless developments were performed for the same length of time in the same solvents. Because of the nature of calibration curves (Figs. 2 and 3) it also would be necessary to compare FID response ratios with the same loads: the variation in response ratios with varying loads has been discussed previously (7,15). Furthermore, rod conditioning can be expected to play some part in determining response ratios, and this also would have to be standardized before successful comparisons could be made. Nonetheless, if developing conditions within a laboratory are standardized and if calibration curves are constructed, a reasonably accurate assessment of the lipid class content of a sample should be obtained. A worthwhile exercise would be to test this by performing an interlaboratory calibration.

Since different Chromarods elicit different FID responses (Figs. 2, 5, 7) it should be possible to eliminate some of the variability in FID

responses by considering each rod as an isolated analytical unit. FID data were compiled in this manner for a comparison between the precision of FID responses after single and double developments (Table 3). Intrarod CVs were obtained from the response data for each Chromarod in the set. By taking the average of the 10 CVs, a mean intrarod CV representative of the rods under each set of developing conditions was obtained (Table 3).

CVs for classes analyzed on the first scan and for PL were improved with double developments in the same solvent system (Table 3). For the other classes, especially TG, the precision was worse after a double development. This may be related to the use of different development times in different solvent systems [Fig. 7(b)]. The second hexane-based solvent system used (98% hexane by volume) was considerably less polar than the first one (80% hexane). It is possible that the redevelopment performed in this system was not quite long enough and that the solvent system was not quite polar enough to ensure a uniform FID response for all scans of these compounds. This would be especially true for TG which is moved the furthest from the point of application in the first development [Fig. 7(a)].

The improvement in CV for PL (Table 3) is exceptional, considering that developed peaks have a lower area [Fig. 7(b)]. The improvement in standard deviation probably is related to the lack of mobility of PL in neutral lipid solvent systems [Fig. 7(a)].

TABLE 3

Mean Intrarod CV of FID Responses from 3 μg of each Lipid Class with 7 Analyses per Compound per Rod, Developed Either Once or Twice

Class	Single development[a]	Double development[a]
HC	13	12
WE	17	16
KET	9	9
FFA	20	16
TG	11	19
ALC	8	13
ST	12	13
PL	11[b]	9

[a]Typical chromatograms are shown in Figs. 6 and 7.
[b]PL remains at the point of application: Fig. 7(a).

TABLE 4

Mean CV of Interrod and Intrarod FID Responses and Ratios (italicized) from Double Developments

Class	Interrod precision		Intrarod precision	
HC	17	*14*	12	*12*
WE	14	*13*	16	*18*
KET	13	–	9	–
FFA	20	*14*	16	*17*
TG	16	*15*	19	*19*
ALC	15	*15*	13	*13*
ST	16	*12*	13	*14*
PL	14	*16*	9	*16*

Inter- and Intrarod Precision

If different rods elicit different responses as a result of the overall characteristics of individual Chromarods, then the use of an internal standard should reduce interrod variability. Similarly, the use of data from individual rods should also reduce the variability in analyses so long as rod conditioning is the same for every development. Table 4 indicates that differences in the characteristics of Chromarods are not the major cause of variability in the Iatroscan-Chromarod system.

Table 4 gives an intercomparison between interrod precision and intrarod precision for the same set of 10 Chromarods. The intrarod precision data is the same as that used for double developments in Table 3. Thus, there are seven analyses per compound per rod. The interrod precision in Table 4 is calculated from the same data set.

The FID responses of some compounds display a deterioration in precision when their areas are normalized to that of the internal standard. For some others the precision is worse if intrarod data is used rather than interrod data. This implies that the magnitude of FID responses for some compounds is independent of that of the internal standard, while for others the differences in FID responses among the 10 rods of a set during any given run is smaller than the variability from run to run. For the latter compounds there is some suggestion of a bimodal distribution of FID responses and of a correlation among the responses of these compounds. Thus, for any particular run, if the peak for WE on the first rod was notably higher than the mean for that compound on the previous run, then not only would the remaining WE peaks on the nine rods be high, but so would the TG and FFA peaks. This distribution was not discernible in the FID responses of the other compounds.

Although no detailed pattern emerges from Table 4, it is possible to make some tentative recommendations on the basis of these data. Since, on average, intrarod precision is at least as good as interrod precision, it should be possible to construct individual calibration curves

over relatively long time periods. This is useful when a large number of samples need to be analyzed, a common requirement in oceanography.

When KET is used as a 'surrogate spike' (21) for estimating the recovery efficiency of lipid classes from seawater samples (3), it cannot be used simultaneously as a reference to compensate for the variability in Iatroscan FID responses. This is because the different response ratios obtained from different loads [Figs. 2 and 3, (7,15)] would necessitate the recovery from seawater samples of identical amounts of KET for any given set of standards. Thus, for the determination of lipids in spiked seawater samples it is better to leave calibration data in integrator area units (3) than to normalize it to a fixed amount of KET (Figs. 2 and 3). Since intrarod precision is not improved by taking data in ratio to the response for KET (Table 4), this is not a drawback.

If only a few samples are to be analyzed, calibration curves can be constructed on the basis of interrod data, especially if a controlled amount of internal standard is added to each Chromarod. This is the most common method used for TLC/FID calibration (6,7,12,15,17). The use of an internal standard improves reproducibility for classes analyzed on the same scan [Table 4, (2,7)]. However, it is advantageous to use the unique partial scanning facility on the Iatroscan when complex lipid mixtures need to be characterized. Since normalization to KET produces the best improvements for interrod data obtained on the same scan (Table 4), an internal standard ideally should be included with every scan.

The most accurate assessment of marine lipid class concentrations should be obtained from a calibration scheme that permits an examination of both run to run and rod to rod differences. In effect this is a means of parameterizing the variability in the FID response originating at the detector stage, and the variability of the FID response that is a result of differences among Chromarods. Unfortunately, even with the most detailed calibration schemes, it is unlikely that the Iatroscan-Chromarod system, in its present design, will ever approach the precision currently enjoyed by other chromatographic techniques. However, recent innovations in the Iatroscan system (22,23) should begin to bridge this gap.

In its present format, the Iatroscan-Chromarod system is likely to be more than adequate for many oceanographic studies. Problems associated withshipboard laboratories, sampling and operationally defined 'fractions' of organic matter (24) are a more serious impediment to the collection of precise data than are most analytical techniques. Detailed analyses of minor organic compounds in seawater often are of secondary importance to oceanographers. Of greater interest is an assessment of the more fundamental interactions between classes of marine compounds and biological and physical processes occurring in the water column.

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Comparison of the Thin Layer Chromatography/Flame Ionization Detection System with Other Methods for the Quantitative Analysis of Liver Lipid Contents in Alcohol-Fed Rats and Controls

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ABSTRACT

In this study, we have examined the feasibility of using the Thin Layer Chromatography/Flame Ionization Detection (TLC/FID) system to evaluate the lipid content of alcohol-induced fatty liver by comparing the results with those from other methods. Various amounts of standards (tripalmitin, phosphatidylcholine and cholesterol) were spotted on Chromarods and scanned either with or without development in a solvent system. The detector responses were significantly greater when the spots were not developed. From the results with developed rods, conversion factors (amount/area) were calculated. These were used for the quantitative analysis of the liver lipids from rats fed a Lieber-DeCarli ethanol diet or a control diet for four weeks. The triglyceride (TG), phospholipid (PL), cholesterol (CH) and cholesterol ester (CE) contents (65.9, 25.4, 2.9 and 6.8 mg/g, respectively) obtained by the TLC/FID system were similar to those observed by other methods (67.9, 27.6, 3.0 and 8.3 mg/g, respectively). The liver lipid content in control rats also was similar to that obtained by other methods (TG, 19.0 vs 20.6; PL, 24.2 vs 21.8, CH, 2.1 vs 2.1 and CE, 1.8 vs 2.6 mg/g). Thus, the magnitude of changes in liver lipid levels due to chronic alcohol ingestion obtained by alternate methods also was found with TLC/FID. The TLC/FID system provides a convenient method for rapid analysis of the extent of fatty liver in alcohol-fed animals.

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INTRODUCTION

During the past several years, we have carried out many investigations to determine whether the fatty liver caused by chronic alcohol ingestion can be prevented by dietary alteration or supplementation (1-4). In these, a gas liquid chromatographic (GLC) method was used to quantitate the lipid contents of liver (1). The triglyceride (TG) content of fatty livers observed by this method was similar to that reported by others who used different analytical procedures (1,5,6). The GLC technique was found to be advantageous not only because it gave reproducible data, but also because it provided the fatty acid composition of lipids, which was necessary to pinpoint the source of TG in fatty liver (diet fat or lipogenesis) and to understand the effect of alcohol ingestion on hepatic fatty acid desaturation (7). However, one of the disadvantages of the GLC method is that long periods of time are necessary for the quantitation of various lipid components. In the present study, we examined whether the Thin Layer Chromatography/Flame Ionization Detection (TLC/FID) system (8), which can analyze up to 10 samples rapidly at one time, can be used to evaluate the extent of fatty liver caused by the chronic ethanol ingestion.

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MATERIALS AND METHODS

Fatty livers analyzed in this study were obtained from rats which were fed a Lieber-DeCarli alcohol diet for four weeks as described earlier (1,2). Control livers were from rats which were pair-fed a Lieber-DeCarli liquid diet in which alcohol was replaced with an isocaloric amount of dextrins (1,2). Isolation of total liver lipids, separation into different classes by TLC, preparation of fatty acid methyl esters and analysis by GLC were carried out as described earlier (1,2). Fatty acid methyl esters were quantitated by using methyl pentadecanoate as an internal standard (1). Hepatic contents of cholesterol (CH) and cholesterol esters (CE) were determined by the o-phthalaldehyde method (9).

The TLC/FID analysis of lipids was carried out using Iatroscan TH-10 Analyzer Mark III (Iatron Laboratories, Tokyo, Japan). The conditions for the storage of Chromarods (Type S-11), spotting the samples and scanning were essentially the same as those used earlier in this and other laboratories (10,11). Peak areas were measured by a Hewlett-Packard 3390 Integrator (Palo Alto, California). The response of TLC/FID to different lipid standards was determined by using the same set of 10 Chromarods. However, during the course of our quantitative analysis of liver lipids, some rods were broken. These

were replaced with rods with properties matching the remaining rods. For the separation of CH, TG and CE fractions from phospholipids (PL), Chromarods were developed in benzene. After scanning to determine the content of neutral lipids, the rods were redeveloped in a solvent system containing chloroform, methanol and water (80:35:3.5, v/v/v) and again scanned to quantitate PL. The lipid standards used in this study were purchased from Applied Science Laboratories (State College, Pennsylvania) and Supelco, Inc. (Bellefonte, Pennsylvania).

RESULTS AND DISCUSSION

In order to determine the liver lipid contents, preliminary studies were carried out to obtain the detector responses of the TLC/FID system to lipid standards (Tables 1-3). Tripalmitin (TP) and phosphatidylcholine (PC) were used as standards for TG and PL, respectively.

Recently, Parrish and Ackman (12) observed that when TP (5 μg) was spotted on Chromarods and scanned without development, peaks of variable shape and area were obtained. Even though development of the rods did not cause any significant mobilization of TP from the point of application, the area observed from the developed spots was appreciably less than that from rods which were not developed (12). In the present study, various amounts of different lipids were spotted on Chromarods and were scanned either with or without the development in a solvent system which caused the spots to migrate (Table 1-3). In the case of TP, the greatest difference in the response between developed and undeveloped spots was observed when the amount applied on the Chromarods was less than 5 μg. At higher concentrations, the responses were similar with or without development (Table 1). With PC and CH, at the various concentrations tested, the responses from developed rods were different from those which were not developed (Tables 2 and 3). The amount that represented unit area (conversion factor) was found generally to be constant in the case of developed rods over a wide range of concentrations of the lipid standards (Tables 1-3). With TP, when concentrations less than 5 μg were analyzed, higher values were observed for this factor (Table 1).

TABLE 1

Effect of Development on Response of TLC/FID to Tripalmitin[a]

Amount analyzed (μg)	Area × 10^{-5}		% Difference	Conversion factor $\frac{\mu g}{area} \times 10^6$
	Developed rod	Undeveloped rod		
2.51	1.51 ± 0.09	3.03 ± 0.20	+102	16.70
4.18	2.72 ± 0.18	5.62 ± 0.35	+107	15.35
6.68	6.75 ± 0.38	8.33 ± 0.32	+ 23	9.90
8.35	9.19 ± 0.36	10.63 ± 0.69	+ 16	9.10
10.02	10.40 ± 0.59	12.03 ± 0.80	+ 16	9.63
13.36	15.91 ± 1.69	15.31 ± 0.71	- 4	8.40
				16.03 ± 0.95 (<5μg)
				9.26 ± 0.66 (>5μg)

[a]Values for the area are given as mean ± SD from determinations by scanning 10 Chromarods with each concentration. Conversion factor was obtained using the area from scanning the developed rods.

TABLE 2

Effect of Development on Response of TLC/FID to Phosphatidylcholine[a]

Amount analyzed (μg)	Area × 10^{-5}		% Difference	Conversion factor $\frac{\mu g}{area} \times 10^6$
	Developed rod	Undeveloped rod		
1.84	1.52 ± 0.19	2.09 ± 0.16	+38	12.1
3.07	2.52 ± 0.21	3.20 ± 0.26	+27	12.2
4.43	3.51 ± 0.35	5.09 ± 0.45	+45	12.6
6.15	4.62 ± 0.24	7.13 ± 0.56	+54	13.3
9.22	7.30 ± 0.49	11.38 ± 0.64	+56	12.6
				12.56 ± 0.05

[a]Values for the area are given as mean ± SD from determinations by scanning 10 Chromarods with each concentration. Conversion factor was obtained using the area from scanning the developed rods.

The various conversion factors obtained from the analysis of the standards were used to quantitate the lipid content in the livers of alcohol-fed or control rats (Tables 4-6). For this purpose, different amounts of total lipids containing various amounts of TG (as judged by the GLC analysis) were spotted and scanned after development. The amount detected by the TLC/FID system occasionally gave values which were higher than those expected from GLC analysis (Table 4). In some experiments, the values from the TLC/FID method were found to be significantly lower than those from the GLC analysis. It is possible that such differences are related to the application of a small volume (1 μl) of the sample on Chromarods. In spite of such variability in the results, the TLC/FID system is useful because several analyses of a sample can be carried out at the same time, and many values for the lipid content are made available. If some values are found to be ambiguous, they can be excluded. In our analysis of the hepatic lipid content in fatty livers, when the relatively higher values (100.4, 143.4 and 114.4) were omitted, the TG content observed by TLC/FID was remarkably close to the value from GLC analysis (62.49 vs 61.96 and 65.94 vs 67.89 mg/g) (Table 4). In experiments with rats fed a Lieber-DeCarli alcohol diet containing glucose (18.6 g/l) for 4 weeks, the degree of fatty livers was reduced because the TG content determined by the GLC procedure was only 31.3 ± 2.9 mg/g (13). With the TLC/FID system, such a reduction in the TG content also was

TABLE 3

Effect of Development on Response of TLC/FID to Cholesterol[a]

Amount analyzed (μg)	Area × 10^{-5}		% Difference	Conversion factor $\frac{\mu g}{area} \times 10^6$
	Developed rod	Undeveloped rod		
1.50	2.07 ± 0.20	2.78 ± 0.36	+25	7.24
3.75	5.14 ± 0.53	7.50 ± 0.50	+46	7.30
5.25	7.50 ± 0.59	10.33 ± 0.97	+38	7.00
7.50	11.15 ± 0.87	14.89 ± 1.42	+34	6.73
11.25	17.52 ± 1.01	21.18 ± 1.29	+21	6.42
15.45	23.00 ± 1.17	30.20 ± 2.58	+31	6.72
				6.90 ± 0.34

[a]Values for the area are given as mean ± SD from determinations by scanning 10 Chromarods with each concentration. Conversion factor was obtained using the area from scanning the developed rods.

TABLE 4

Comparative GLC and TLC/FID Analyses of Liver TG Contents in Rats Fed a Lieber-DeCarli Alcohol Diet[a]

Amount analyzed (μg)	Amount observed TLC/FID (μg)	mg TG/g liver TLC/FID	mg TG/g liver	
			TLC/FID	GLC
2.67	4.33 ± 0.66	100.40*		
5.35	5.08 ± 0.39	58.81		
8.02	8.30 ± 0.23	64.13	70.07 ± 17.13	61.96 ± 3.24
11.59	12.19 ± 1.75	65.21	62.49 ± 2.8 [b]	
14.26	14.22 ± 0.69	61.80		
5.88	5.93 ± 0.30	68.48		
11.76	24.85 ± 1.88	143.42*		
14.12	10.18 ± 2.53	48.98	86.92 ± 34.9	67.89 ± 5.52
17.65	18.88 ± 2.52	72.63	65.94 ± 11.5[b]	
20.00	21.70 ± 0.90	73.67		
23.53	39.64 ± 3.17	114.37*		

[a]Results from the fatty livers of two different alcohol-fed rats are given. TLC/FID analysis with each concentration was carried out with 10 Chromarods. Values are mean ± SD.

[b]These values were calculated by omitting the higher values marked by an asterisk.

TABLE 5

Comparative GLC and TLC/FID Analyses of Liver TG Contents in Rats Fed a Lieber-DeCarli Alcohol Diet Supplemented with Glucose[a]

Amount analyzed (μg)	Amount observed TLC/FID (μg)	mg TG/g liver TLC/FID	mg TG/g liver	
			TLC/FID	GLC
3.23	2.70 ± 0.18	26.96		
6.46	4.03 ± 0.42	20.08	23.19 ± 2.84	31.30 ± 2.94
9.69	6.80 ± 0.17	22.60		
12.93	9.27 ± 0.61	23.12		

[a]Analysis of liver TG content was carried out in rats which, for 4 weeks, were fed a Lieber-DeCarli alcohol diet supplemented with glucose (18.6 g/l) (13). TLC/FID analysis with each concentration was carried out with 10 Chromarods. Values are mean ± SD.

TABLE 6

Comparative GLC and TLC/FID Analyses of Liver TG Contents in Rats Fed a Lieber-DeCarli Control Diet[a]

Amount analyzed (μg)	Amount observed TLC/FID (μg)	mg TG/g liver TLC/FID	mg TG/g liver	
			TLC/FID	GLC
0.91	1.39 ± 0.32	7.65		
1.82	3.31 ± 0.70	9.13	9.10 ± 2.1	5.01 ± 0.01
2.73	4.07 ± 0.53	7.47		
3.64	8.77 ± 1.54	12.08		
2.03	2.56 ± 0.17	24.05		
4.06	4.12 ± 0.26	19.29	20.58 ± 2.39	19.04 ± 1.13
6.09	6.00 ± 0.61	18.74		
8.12	8.63 ± 0.80	20.23		

[a]Results given are from separate livers from two rats which were fed the Lieber-DeCarli control diet. With each concentration, 10 Chromarods were scanned. Values given are mean ± SD.

observed, although the values obtained were lower compared to those from the GLC method (23.2 ± 2.8 mg/g) (Table 5).

When livers of control rats were analyzed, the TLC/FID method yielded a value markedly smaller than that found in fatty livers (9.1 mg/g) (Table 6). However, this value was about twice that observed by the GLC method (5.01 mg/g). In spite of this difference, it is clear that the use of TLC/FID shows the production of fatty liver in alcohol-fed rats as compared to controls. In some control livers which contained a higher level of TG (19.04 mg/g), the results from the TLC/FID system and GLC were almost identical (Table 6).

Scans of Chromarods also enabled us to quantitate the hepatic content of PL, CH and CE (Table 7). Several studies have shown that the response factors for CH and CE are somewhat similar (11,14-16). Hence, in the present study, the conversion factor generated from the analysis of cholesterol also was used for the estimation of the CE content of livers. Liver PL contains not only PC but also phosphatidylethanolamine (PE) and small amounts of sphingomyelin (SP). Although the response factors for PE and SP are similar and somewhat less than those for PC (17), we used the conversion factor obtained for PC to quantitate the total PL. In spite of these limitations, the amount of PL, CH and CE/g liver observed by the TLC/FID system was similar to the corresponding value from other methods in the case of both fatty liver and control liver (Table 7).

The results obtained in the present study demonstrate that the TLC/FID system offers a rapid means to evaluate the extent of alcoholic fatty liver by analyzing the TG content of total lipids. It also provides a quantitative measure of the PL, CH and CE content of liver. The conclusions drawn by using other analytical methods regarding the quantitative distribution of lipid components of liver and changes due to the chronic ingestion of ethanol or dietary supplementation also were obtainable with the TLC/FID system. Therefore, this new analytical tool

TABLE 7

Lipid Content of Livers in Rats Fed Alcohol and Control Diets[a]

Lipid class	Controls		Alcohol-fed	
	Other methods	TLC/FID	Other methods	TLC/FID
	mg/g liver			
Triglyceride	19.04 ± 1.13	20.58 ± 2.39	67.87 ± 5.52	65.94 ± 11.5
Phospholipid	24.15 ± 1.55	21.84 ± 4.4	27.64 ± 1.72	25.38 ± 2.43
Cholesterol	2.10 ± 0.01	2.14 ± 0.19	2.99 ± 0.02	2.93 ± 1.00
Cholesterol ester	1.8 ± 0.01	2.55 ± 0.37	8.3 ± 0.64	6.79 ± 1.66

[a]Values given are mean ± SD. Other methods of analysis were GLC for TG and PL and the o-phthalaldehyde method for cholesterol and CE. The amount of fatty acid methyl esters generated from TG approximate the wt of TG. From the amount of fatty acid methyl esters obtained from PL, the PL content was calculated using the molecular weight of dipalmitoyl-PC.

also can be useful for the quantitative determination of the components of lipids from other sources.

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Quantitating Heart Lipids: Comparison of Results Obtained Using the Iatroscan Method with Those from Phosphorus and Gas Chromatographic Techniques

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ABSTRACT

The precision and accuracy of the Iatroscan method was evaluated by comparing the results obtained with established phosphorus and gas chromatographic techniques. A complete lipid class analysis of rat heart lipids was chosen in order to evaluate the performance of the Iatroscan method for biological samples which contained both neutral lipids and phospholipids. A partial scan and repeat development with chloroform/methanol/water (68.5:29:2.5) was introduced to achieve consistently good separations of the phospholipids on the Chromarods in the Iatroscan method. The results showed that the precision of the Iatroscan method for some lipid classes was comparable to that of phosphorus or gas chromatographic techniques, while for other lipid classes it was lower. Compared to the data obtained using the phosphorus method, the Iatroscan data were generally similar, while the gas chromatographic method generally gave lower values. These findings, together with the advantages of time required for analysis, size of sample, and universality of detection, suggest that the Iatroscan is a valuable complementary method for complex lipid analyses.
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INTRODUCTION

The use of the Iatroscan method in the analyses of lipid has increased markedly in the past decade (1). With this method, lipid mixtures are separated on silicic acid coated quartz rods and then quantitated by passing the rods through a flame ionization detector. The two concerns about this comparatively new method are the quality of separations and the precision and accuracy of the results. Several studies have been conducted in which mixtures of known composition were analyzed (2-7). Unfortunately, such mixtures usually contained about equal proportions of each component, and so mixtures of widely different concentrations of components, a situation normally found in biological samples, have not been tested. The exception has been the analyses of the neutral lipids and total phospholipid of plasma (8-11), serum lipoproteins (12) and skin surface lipids (13). In these publications, the results by the Iatroscan method were compared with those obtained by traditional methods such as chemical (8-10,12), gas chromatographic (GC) (11) and thin layer chromatographic (TLC)-densitometric (13).

In the present study, the complete lipid profile of rat heart, including all the neutral lipids and phospholipids, was resolved using improved developing solvents and quantitated by the Iatroscan method. The precision and accuracy of the Iatroscan results are compared with those obtained by two well established techniques, TLC followed by phosphorus analysis and TLC followed by GC analysis using an internal standard.

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MATERIALS AND METHODS

Preparation of Heart Lipids

Hearts were taken from male rats fed from weaning on isocaloric diets, containing 5, 10 or 20% fat, for 12 weeks. The dietary fat consisted of a 9:1 mixture of olive oil and corn oil whose fatty acid composition was 10.7% 16:0; 0.6% 16:1; 3.5% 18:0; 74.5% 18:1; 9.3% 18:2; 0.3% 18:3; 0.5% 20:0; 0.3% 20:1. The rats were killed by CO_2 asphyxiation and the hearts were removed, weighed, pulverized immediately at dry ice temperature and the total lipids extracted with chloroform/methanol (2:1) as described previously (14). The total lipids from two hearts per diet were combined, taken up in a 10-ml volumetric flask with chloroform/methanol (2:1), and divided into 10 1-ml aliquots (ca 5 mg/ml) sealed in auto-sampler vials and kept at -20 C until analyzed.

Phophorus Method

The analyses were performed using five 1-ml aliquots per diet. The phospholipids were resolved by two-directional TLC as described previously (15), using chloroform/methanol/28% aqueous ammonia (65:25:5) in the first direction, and chloroform/acetone/methanol/acetic acid/water (50:20:10:15:5) in the second direc-

tion. Spots corresponding to the phospholipids were visualized under UV light after spraying with 2',7'-dichlorofluorescein, removed, and analyzed for phosphorus according to Kates (16). Absorbances were recorded at 680 mμ in acrylamide cuvettes (Sarstedt, St. Laurent, Quebec) using a Zeiss Model PMQ II UV Spectrophotometer. The content of each phospholipid was then calculated based on an average molecular weight obtained from the GC data of that particular phospholipid.

Gas Chromatographic Method

The analyses were performed using three 1-ml aliquots per diet. All the phospholipids and neutral lipids were resolved on one TLC plate as described previously (15). Spots corresponding to all the cardiac lipid classes were scraped off into 20-ml culture tubes containing 0.5 ml benzene. Methyl heptadecanoate (Nu-Chek-Prep, Elysian, Minnesota) was added as internal standard to all lipid classes, except cholesterol, so that the concentration of the internal standard was ca 20% of the total methyl esters. β-Sitosterol (ICN Nutritional Biochemicals, Cleveland, Ohio) was used as an internal standard for the quantitation of cholesterol. Lipids were transesterified with anhydrous HCl/methanol (5% HCl by weight). The methylated products were purified by TLC using the developing solvent hexane/diethyl ether/acetic acid (85:15:1) and taken up in CS_2 for analysis by GC. To avoid oxidation of polyunsaturated fatty acids, all reactions and manipulations were performed under nitrogen.

The Hewlett-Packard Model 5380A GC was equipped with flame ionization detectors, digital integrator and automatic sampler (Hewlett-Packard Model 7671A). Methyl esters were analyzed using a flexible fused silica capillary column (30m × 0.25mm i.d.) coated with 0.25 μm Carbowax 20M (Chromatographic Specialties Ltd., Brockville, Ontario). The GC was temperature programmed from 195 to 215 C at 2 C per min after an initial 20-min isothermal run. The sterols were analyzed using a flexible fused silica capillary column (12 m × 0.25 mm i.d.) coated with 0.25 μm SP 2100 (Hewlett-Packard, Ottawa, Ontario), operated isothermally at 240 C. The other parameters were: injector and detector temperatures 250 C; split ratio 5:1; helium flow through column 1.1 ml/min; nitrogen make up flow 29 ml/min; hydrogen flow 30 ml/min; air flow 240 ml/min; and attenuation 4. An average molecular weight was calculated based on the fatty acid composition of each lipid class. The value was used to convert the molar content of each lipid class to a weight.

Iatroscan Method

The analyses were performed using two 1-ml aliquots per diet. Each vial of heart lipids was taken to dryness with a stream of nitrogen, and 150 μl of methyl heptadecanoate (1 μg/μl) in chloroform/methanol (2:1) was added. For each of the aliquots, 1 μl of the solution was spotted on each of eight silica gel Chromarods (type S). The set of rods was equilibrated before each development for 5 min in a 55% humidity chamber (41% H_2SO_4) and 5 min over the developing solvent. The neutral lipids were developed in 1,2-dichloroethane/chloroform/acetic acid (92:8:0.1) (17) to a height of 11 cm, and the rods were partially burned to Rf=0.15. The polar lipids then were resolved by developing the Chromarods twice up to 10 cm in the solvent chloroform/methanol/water (68.5:29:2.5), with an air drying and another equilibration between developments. The second scan was a complete burn of the rods.

The Iatroscan TH-10 Analyzer, Mark II (Iatron Labs., Tokyo, Japan; Canadian distributor: Technical Marketing Associates, Mississauga, Ontario) was equipped with a flame ionization detector and connected to a Hewlett-Packard 3390A integrator. The hydrogen flow was 160 ml/min, air flow 2 l/min and scanning speed 0.42 cm/sec. The response correction factors calculated by analyzing a standard mixture containing an equal amount of methyl oleate (1.000) were triolein (0.721), cholesterol (0.439), diphosphatidylglycerol (1.633), phosphatidylethanolamine (1.589) and phosphatidylcholine (0.999). The response factors for the minor lipid classes were taken as unity.

Statistics

Because of the differences in experimental protocol from method to method and because of the possibility of heterogeneity of variance, analyses of variance were applied to the data from each method separately. The estimates of variance components were derived from these analyses of variance, using the expected mean squares. The fact that several Chromarods can be used in one determination complicates comparisons of precision. The precision estimates presented here are based on the use of eight Chromarods per determination. However, to calculate the appropriate standard error for fewer or more rods, the reader need only use the estimates of variance components shown in Table 1.

RESULTS AND DISCUSSION

Qualitative Analysis

All of the neutral lipids and phospholipids were well resolved for the phosphorus and GC

TABLE 1

Determination of Heart Lipid Classes Using Phosphorus Analysis, a Gas Chromatographic Technique or the Iatroscan Method

Method	% Fat in Diet	Heart lipid classes[a] (mg/g wet tissue)								
		CE	TG	C	DPG	PE	PI	PS	PC	SP
Phosphorus	5	–[b]	–[b]	–[b]	2.5	6.2	1.1	0.5	8.1	0.9
(P)	10	–	–	–	2.8	6.0	1.1	0.6	8.1	0.6
(n=5)	20	–	–	–	2.9	6.5	1.3	0.9	8.8	0.7
Gas chromatography	5	0.2	4.9	1.0	2.3	5.6	0.8	0.4	8.0	0.5
(GC)	10	0.3	4.0	1.0	2.2	5.1	0.8	0.3	7.9	0.5
(n=3)	20	0.2	5.8	0.9	2.1	5.6	0.7	0.3	7.7	0.6
Iatroscan	5	0.2	5.0	1.3	3.2	7.0[c]	–[c]	0.5	7.3	1.0
(I)	10	0.1	3.7	1.2	2.6	5.8	–	0.5	6.7	0.7
(n=16)	20	0.2	6.4	1.3	3.5	8.5	–	0.6	9.7	0.8
Variance components[d]										
P Among diets		–	–	–	.045**	.041*	.005	.033*	.145**	.011
Within diets		–	–	–	.022	.036	.062	.050	.115	.019
GC Among diets		.002	.596	-.004	-.030	.071*	.001	-.003	.021	-.001
Within diets		.003	.552	.016	.123	.039	.006	.012	.021	.019
I Among diets		.004*	1.874**	-.023	.121**	1.526**	–	-.001	2.408**	.001*
Among aliquots[e]		.001	.021**	.063**	.198**	.547**	–	.005	.249**	.008*
Among rods[e]		.002	.044	.015	.079	.670	–	.027	.276	.023
Standard error[f]										
P		–	–	–	.15	.19	.25	.22	.34	.14
GC		.06	.74	.12	.35	.20	.07	.11	.15	.14
I		.03	.16	.25	.46	.79	–	.09	.53	.11

[a]CE, cholesteryl esters; TG, triglyceride; C, cholesterol; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SP, sphingomyelin.

[b]–, No phosphorus analysis possible.

[c]PI included in PE results.

[d]Variance components and standard deviations were calculated from the analysis of variance. Statistical significance at the 5% (*) and 1% (**) level of the corresponding mean squares was based on the appropriate F-ratios.

[e]Among aliquots and among rods within diets.

[f]The standard error of the reported value of an aliquot.

methods based on prior separation of the lipid classes by three-directional TLC (15). The lipid spots were quantitatively removed from the TLC plate, methylation was performed in the presence of the silica gel to minimize loss of lipids, and all manipulations and reactions were carried out under nitrogen.

Resolution of all the heart lipids by the Iatroscan method was achieved by a combination of developing solvent mixtures. The neutral lipids and internal standard (methyl ester) were separated using 1,2-dichloroethane/chloroform/formic acid (92:8:0.1) (17), in order to include cholesterol in the first scan and to avoid the use of an additional solvent, like acetone, to resolve cholesterol from phospholipids (18). The phospholipids were resolved by developing the rods twice in chloroform/methanol/water (68.5:29:2.5) to 10 cm, which improved the resolution of PC and SP, and provided a larger gap between PE and PC, allowing for the enhanced separation of PI and PS (Fig. 1). However, PI could not always be resolved from PE, and for this reason PE and PI were combined in the Iatroscan data (Table 1). Our most recent evidence indicates that differences in fatty acid chain length and unsaturation, present particularly in phospholipids (Table 2), cause peak broadening and poorer resolution on Chromarods (19). The resolution of lipids on Chromarods also could be improved by use of an applicator spotting very narrow bands (20), and modification of the detector to discriminate closely resolved peaks.

Quantitative Analysis

This is the first report in which all the phospholipids in a biological sample were quanti-

tated using the Iatroscan method and compared to the phosphorus and GC methods. The results of the quantitative analyses of rat heart lipids using the three methods are shown in Table 1.

The amount of heart lipid required per analysis of all the lipid components by the phosphorus and GC methods was ca 5 mg. This amount represented about 1 μg phosphorus or 25 μg of material for a lipid class present at 0.5% of the total. On the other hand, the Iatroscan method required only about 33 μg of total lipids per analysis. Subsequent to this study it was pointed out by Patterson (21) that the ion collector in most Iatroscan instruments may be adjusted in height above the Chromarods. We experienced a 4-fold increase in detector response by adjusting the ion collector 1.7 mm above the rods. Thus, the amount of lipid required per analysis is further reduced.

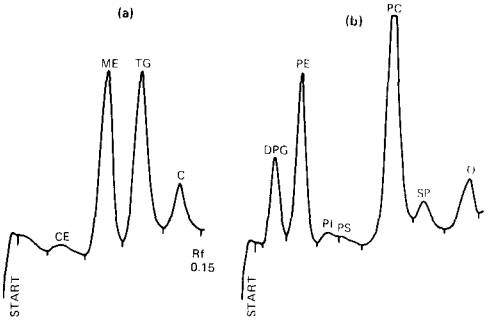

FIG. 1. Consecutive development of rat heart lipids on the same Chromarod with the Iatroscan method. First development of neutral lipids and internal standard (Fig. 1a) was in 1,2-dichloroethane/chloroform/formic acid (92:8:0.1), followed by partial scan from the top down to Rf 0.15. Second development of phospholipids (Fig. 1b) was twice to a height of 10 cm in chloroform/methanol/water (68.5:29:2.5), followed by a complete burn of the Chromarods. O = point of application of sample; start = start of scan.

The comparisons among methods involved an evaluation both of accuracy and precision. The variance components of the individual lipid classes for each method (Table 1) were used to compare precision. The among diet entries reflect any diet difference specific to the study. Large differences in these entries indicate serious discrepancies in reported values for at least one diet; small differences also could be attributable to the difference in replication. For the determination of phospholipids, the phosphorus method has been considered by many, including ourselves, to be the most accurate method (16, 22,23). Hence, the results from the phosphorus analysis were used in this study as a standard by which to assess the accuracy of the GC and Iatroscan methods.

In general, analyses by the GC method resulted in slightly lower values for all the phospholipids than for those obtained with the phosphorus analysis (Table 1). The reason is not apparent. Oxidation of polyunsaturated fatty acids in the phospholipids (Table 2) during sample preparation was ruled out, because all manipulations were performed under nitrogen.

TABLE 2

Fatty Acid Composition of the Major Rat Heart Lipid Classes
Average of the Three Diets Containing 5, 10 or 20% Fat (3 analyses/diet)

	Lipid classes[a]						
	TG	DPG	PE	PI	PS	PC	SP[b]
14:0	0.8	0.2	0.1	0.3	0.6	0.1	0.6
16:0	17.6	1.4	6.3	3.6	4.4	14.6	15.6
16:1	2.6	1.5	0.2	0.8	1.5	0.4	1.3
18:0	4.3	1.5	27.2	42.0	49.3	28.7	20.2
18:1 n-9	61.8	14.9	9.6	13.3	6.4	9.2	6.2
18:1 n-7	3.9	6.2	3.9	1.8	1.0	6.0	1.0
18:2 n-6	6.9	63.7	2.9	2.7	1.2	5.2	1.5
18:3 n-3	0.1	0.1	0.1	0.1	0.3	0.1	–
20:4 n-6	1.1	1.5	27.3	30.3	11.0	31.9	–
22:4 n-6	–[c]	0.2	1.6	0.6	3.0	0.5	–
22:5 n-6	–	2.3	6.6	1.2	10.7	1.1	–
22:5 n-3	–	0.1	0.5	0.1	0.4	0.2	–
22:6 n-3	–	1.4	7.3	0.8	8.5	1.3	–

[a]For abbreviation of lipid classes see Table 1, Footnote a.

[b]Sphingomyelin (SP) contains also: 15.4% 20:0; 2.7% 21.0; 11.5% 22:0; 0.4% 22:1; 4.7% 23:0; 1.9% 23:2; 8.7% 24:0; 7.5% 24:1.

[c] (-), non-detectable levels.

Preferential on-column degradation of polyunsaturates, well known to occur with stainless steel capillary columns (24), was not anticipated with the fused silica capillary columns (25). However, studies are now in progress to evaluate these columns for possible requirements of response factors or a different choice of internal standard (22).

The precision of the GC method was high and generally similar to that of the phosphorus method (Table 1), although the ratio of the within diet variance component of the former to the latter ranged from 5.6 (DPG) to 0.1 (PI). Estimates of precision of the GC method exceeded 0.2 only for the triglycerides (TG). However, despite the high precision, only 1 (PE) of the 4 (DPG, PE, PS, PC) significant diet differences indicated by the phosphorus method was identified by the GC method. Of course, this result may be due in part to the larger number of replicates used with the phosphorus method.

The precision of the Iatroscan results depend on two components, the variance among aliquots and among rods within aliquots. If the latter dominates, precision can be improved considerably by using more than one rod. If the former dominates, however, precision can best be improved by obtaining measurements on more than one aliquot. The results in Table 1 show that, for the major subclasses of the phospholipids, both components are considerably larger than the within diet components of the other two methods, indicating lower precision for the Iatroscan method. The significant F-ratios designated for the among aliquot component for a number of lipid subclasses simply means that the estimate for that component is significantly greater than zero. The possibility that the within aliquot component was due to systematic differences among rods was examined. However, analyses (not shown here) indicated that the component was essentially random with differences among rods varying considerably from aliquot to aliquot. We observed a similar result earlier when looking at different sets of rods used on the same sample (7).

Because it was anticipated that the Iatroscan precision would be lower, it was decided to use eight Chromarods in measuring each aliquot to reduce the standard error associated with each aliquot measurement. The standard errors presented in Table 1 show that this approach was at least partially successful in bringing the precision of the Iatroscan method into line with the other two. However, because of the magnitude of the among aliquot component, especially with PE and PC, it would be necessary to include measurements from several analyses in each reported value to obtain comparable precision. The additional time and material would be no problem.

There was considerable variation in the estimates of precision of the three methods, with the Iatroscan method actually appearing preferable for some subclasses. The variation did not appear to be dependent on the relative concentration of the lipid class or its functional group. In spite of the variation in the Iatroscan results, three of the four differences found to be significant with the phosphorus method also were found to be significant with the Iatroscan method. It may be noted, however, that the Iatroscan method did give some large differences among diets, especially for TG, PE and PC. It was more likely that these large differences arose from problems with the accuracy of the method for these subclasses than with any heterogeneity in the aliquots tested.

Estimates of precision and/or accuracy were reported in several studies in which the neutral lipids and total phospholipids of plasma (8-11), serum lipoprotein (12) and skin surface lipids (13) were analyzed by the Iatroscan method and compared to chemical (8-10,12), GC (11) or TLC-densitometric (13) results. In three reports the chemical and Iatroscan results were reported to be similar both in precision and accuracy (8-10), particularly with the use of an internal standard (10). On the other hand, Mills et al. (12) claimed to obtain better precision for chemical methods than for the Iatroscan in the analysis of serum lipids. In agreement with the present study, Mares et al. (11) claimed better precision for the GC than for the Iatroscan method. However, in their study (11), the accuracy of the plasma lipid analysis could not be assessed because it lacked an independent method to establish the accuracy of the GC method. Finally, a comparison of the results of TLC-densitometry with the Iatroscan method indicated that the latter gave better precision (13), although Christie (22), who supplied no data, claimed the opposite. It is difficult to determine if the differences in the estimates of precision and accuracy reflect real differences between methods. From the personal experience of the authors, it appears that the Iatroscan method gives a wide variation in results, and the accuracy can be improved markedly by use of internal standards (7) and multiple scan with redevelopment analyses. Improvements in sample application (20) and detector design (21) may increase the precision and accuracy of the Iatroscan method.

The results of this study clearly showed that, considering the amount of sample required,

time of analysis, universality of the detector and fairly good precision and accuracy, the Iatroscan method is a valuable tool, complementing other techniques in the analysis of complex tissue lipid mixtures.

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The Use of the Iatroscan TH-10 Analyzer to Quantify Total Lipids in a Variety of Sample Types and Lipid Classes in Human Gallbladder Bile

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ABSTRACT

Two methods for the measurement of total lipid weight in biological and geological samples and the major lipid classes in human gallbladder bile using the Iatroscan TH-10 analyzer are described. Total lipid determination involves the application of small (5 μl) volumes to Chromarods, focusing of the sample into one band by partial development in chloroform-methanol (1:1), and quantification by flame ionization detection (FID). The response variation between different sample types did not affect the linearity of response, allowing a reproducibility of ±10% of the mean or better for samples ranging from 0.5 to 32 μg. Total lipid determinations in 10 samples could be performed in 30 min. The three major components of human gallbladder bile (cholesterol, phospholipids and bile acids) also were quantified with the Iatroscan. Samples focused on Chromarods were separated using a double development scheme in two solvent systems. All three components exhibited a linear response over the range of 0.25 to 8 μg. The repeated scanning of rods required at concentrations greater than 3 μg did not affect linearity of response. Samples from 10 patients could be processed in less than one hr. Several techniques are discussed to increase reproducibility when performing quantitative lipid analysis with the Iatroscan.

Lipids 20:542-545, 1985.

INTRODUCTION

In lipid analysis, thin-layer chromatography (TLC) has been a valuable analytical tool for the separation of lipids. Quantification, however, has been more difficult, due to the time required (i.e. dry weight measurements) or the variability in precision (densitometric measurements). The development of a coupled system utilizing TLC with flame ionization detection (FID), the Iatroscan Analyzer, has been an important advance in the quantitative analysis of lipids (see Ackman [1] for review). Combining the resolution capabilities of thin-layer chromatography with the sensitivity of flame ionization detection, the Iatroscan presents a versatile instrument which has been used in a number of applications from clinical (2) to environmental (3).

We have developed two methods which take advantage of both the quantitative and rapid sample processing abilities of the Iatroscan. In the first method, a quantitative measurement of the total lipid present in a sample is performed without separation of lipid classes. Instead, a focusing step is utilized to ensure that the entire sample is quantified as a single band on the silica covered quartz rod. Taking advantage of the small sample size required for quantitation by FID, less than μg amounts of almost any lipid can be determined rapidly.

The second method relies on the combined separation and quantification abilities of the Iatroscan for a rapid measurement of cholesterol, total bile salts and total phospholipids in a single sample of human gallbladder bile. While several other methods presently exist for the measurement of these components either by colorimetric (4) or enzymatic (5) assay, each component still must be quantified separately. Our method utilizes a two-stage chromatographic separation on Chromarods, with accompanying FID quantitation of each component without prior isolation.

MATERIALS AND METHODS

Initial reports have described the chemicals, preparation of lipid standards and Iatroscan operating conditions for each method (6,7), and therefore only a brief procedural outline will be presented. For total lipid analysis, samples representing several sources were examined. These included purified olive oil (triacylglycerol), olive oil-phosphatidylcholine mixtures (2:3, w/w), solvent extractable lipids of a marine sediment and the total solvent extractable lipids of fish. After initial determination of total lipid weight gravimetrically, samples were redissolved in a mixture of chloroform-methanol (1:1), diluted and stored at −20 C under nitrogen until use.

After activation of Chromarod S-II rods by passage through the hydrogen flame, lipid samples were spotted using a 5 μl Wiretrol (Drummond Scientific Co.) and dried, noting

the distance of solvent spreading from the point of sample application. The rods then were placed in a developing tank containing chloroform-methanol (1:1) and the solvent front allowed to migrate slightly above the uppermost point of sample application (ca. two cm). This focused the entire sample as one band on the Chromarod. Then the rods were dried for 5 min at 100 C to remove all traces of solvent and scanned at a speed of 30 sec per rod with peak area integration performed by a Hewlett-Packard 3390A integrator.

In our second application of the Iatroscan, gallbladder bile obtained from patients undergoing elective cholecystectomy was separated into its component bile salts, cholesterol and phospholipids and compared to a prepared bile salt standard mixture (8). Fresh human bile was collected from patients, homogenized and diluted with chloroform-methanol (1:1) and stored at −20 C. Any bile proteins which precipitated during solvent addition were removed by centrifugation and the clear supernatant used for analysis. The bile salt standard mixture included glycochenodeoxycholate (44.9%), taurocholate (17.9%), taurodeoxycholate (27.3%) and glycocholic acid (9.9%). These bile salts were combined with cholesterol and egg phosphatidylcholine in chloroform-methanol (1:1) and the mixture used to standardize the integrated area response prior to human gallbladder bile analyses.

To compare human bile samples to the standard bile mixture, samples of each were spotted on type S Chromarods in 5 μl aliquots using a Wiretrol and focused as described above. Double development in two solvent systems was required to separate phospholipids, cholesterol and all bile salts as three distinct bands. The first system consisted of chloroform/light petroleum/methanol/acetone (60:20:10:10) and the solvent front run to 8-10 cm. After drying the rods (5 min at 100 C), the second system containing acetone/water (50:50) was used and the solvent front run to 5 cm. Chromarods then were dried as above and scanned.

RESULTS

Total lipid analyses for dilutions of each of the four lipid samples are shown in Figure 1. Five replicates of each dilution were spotted and focused on Chromarods and then quantified on the Iatroscan. Regression analysis of each set of samples indicated an identical linear decrease in peak area (slope = 0.92 ± 0.01) over the concentrations shown. Below these concentrations (approximately 0.3 μg per sample) non-linear decreases were seen

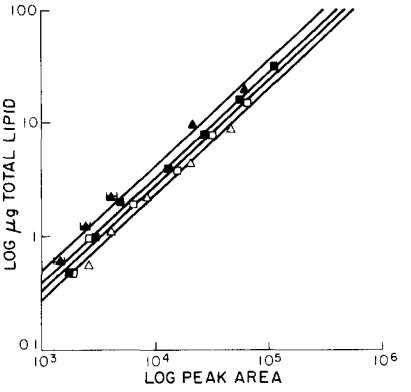

FIG. 1. The relationship between peak are and mass of total lipid. Samples were applied to rods in 5 μl aliquots, focused using chloroform-methanol (1:1), dried at 100 C for 5 min and scanned. Points are the mean of 5 measurements at each dilution: olive oil (□), olive oil-phosphatidylcholine (40:60, wt/wt) (■), marine sediment lipids (△), total fish lipids (▲). Error bars not shown are less than symbol area.

which have been found by other investigators attempting to quantify low lipid concentrations (9). Flame ionization response previously has been shown to vary as much as 2.8 times between equal concentrations of different lipids (10,11), with polar lipids generally eliciting a higher response. The differences in lipid class composition among the diverse samples we examined showed a similar effect (Fig. 1), resulting in increased or decreased area measurements which were dependent upon lipid composition. These differences, however, did not affect the linearity of the total lipid determinations. By using a standard of known composition similar to the lipid sample being measured, quantitative results could be achieved reproducibly from any number of diverse sources.

The separation of cholesterol, bile salts and egg PC in the standard mixture using the separation scheme developed for Chromarods is illustrated in Figure 2. After spraying the TLC plate with H_2SO_4 and charring at 120 C, it can be seen that cholesterol and bilirubin migrate with the solvent front in the first chromatographic system, leaving the bile salts and phospholipid at the origin. In the second system, bile salts travel with the solvent front, the polar phospholipids remaining at the origin. Using this double separation scheme, all three bile components (cholesterol, bile salts and phospholipids) could be quantified in the Iatroscan without prior treatment in less than one hr. A standard curve generated over the range of 0.25 to 8 μg for the standard bile salt

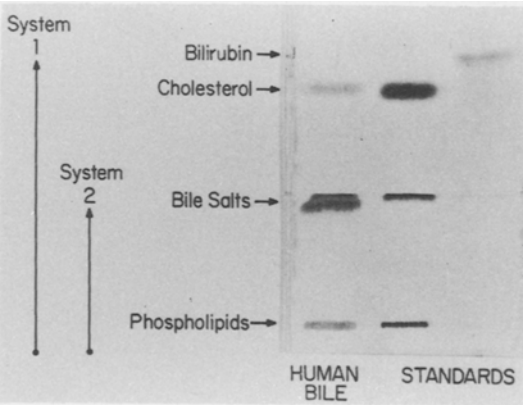

FIG. 2. The separation of bilirubin, cholesterol, bile salts and phospholipids by the two solvent systems used for Chromarods. System 1: chloroform-light petroleum-methanol-acetone (60:20:10:10) developed to 8 cm. System 2: acetone-water (50:50) developed to 5 cm. Spots were visualized by charring (40% sulfuric acid at 120 C for 5 min).

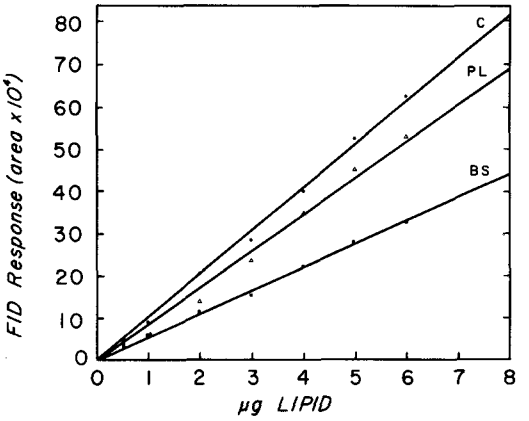

FIG. 3. Standard curve for increasing concentrations of cholesterol (•), egg PC (△) and the bile salt standard mixture (■). The sum of the initial and residual peak areas from consecutive scans was used to calculate sample concentrations above 3 μg per sample. Each point represents the mean of 6 determinations.

mixture is shown in Figure 3. Concentrations greater than 3 μg per rod required 2 to 3 scans to remove any residual bile salts completely. In these cases, peak areas from consecutive scans were then summed to calculate the total sample concentration. Repeatedly scanning the rods (over 170 times) did not appear to affect the resolution or cause the broadening or tailing of peaks. Although regression analyses indicated a linear decrease in response through the origin, prior experience has shown us that non-linear decreases often occur below 0.25 μg lipid.

DISCUSSION

We have utilized the Iatroscan analyzer to rapidly quantify total lipids and the major components of human bile. As an alternative to gravimetric methods, total lipid measurements on the Iatroscan do not require solvent evaporation and multiple weighings, avoiding both oxidation and photodegradation. Since small amounts of lipid (typically less than 1.0 μg) are required and 10 determinations can be made in as little as 10 min, a sensitive measurement of lipid concentration can be made rapidly from diverse sources. By rapidly quantifying the three major components of human bile, the Iatroscan also may have predictive value. According to Carey and Small (12), the total lipid concentration plus the bile salt:phospholipid ratio are the predominant determinants in cholesterol gallstone formation. Using the Iatroscan, the rapid quantification of the major bile components and total lipid over a wide range of clinical concentrations is possible. Such information may be useful in assessing an individual's risk in developing cholesterol gallstones.

In both of the methods discussed, we have used several techniques to ensure maximum reproducibility. These techniques also apply to other applications of the Iatroscan for quantitative lipid analysis. First, the choice of spotting solvent and sample volume can drastically affect reproducibility when spotting on Chromarods by hand. We obtained the best results from spotting samples in chloroform-methanol (1:1) using spotting volumes of 5 μl (6). Throughout the handling and storage of lipid samples, solvent evaporation should be minimized to reduce the concentration effects of solvent loss on lipid samples. Second, all lipid samples should first be focused at one point using chloroform-methanol (1:1) before chromatographic separation to prevent peak splitting or tailing. Third, solvents also should be evaporated completely (generally 5 min at 100 C) prior to scanning or the use of additional solvent systems. Finally, if samples are known to contain inorganic salts, steps must be taken to minimize their accumulation on Chromarods because reproducibility is ultimately affected. If samples containing salts are applied to Chromarods, salts should be removed between sample sets by rinsing or dipping the rods in an aqueous solution for several minutes. We have used both distilled water and a mixture of distilled water-methanol (9:1) with equal success. After dipping, rods can be dried, reactivated

and reused without overnight acid cleaning. The use of the above techniques, in addition to those recommended by the manufacturer, should reduce much of the variation encountered by investigators during first time use of the Iatroscan.

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Quantitative Class Separation of Coal Liquids Using Thin-Layer Chromatography with Flame Ionization Detection

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ABSTRACT

The paper illustrates the use of TLC/FID for the separation of heavy distillates and is a summary of work done by our group over the last few years and published in detail elsewhere. For those who are unfamiliar with fossil fuel analysis, the basic terms and methods are explained with emphasis on the merits and disadvantages of TLC/FID as compared to column and solubility separations. Also, problems with the determination of concentration/response dependence and with absolute detector calibration are outlined.
Lipids 20:546-551, 1985.

INTRODUCTION

In fossil fuel chemistry, information on the so-called "class distribution" of various materials is of major importance, because it gives a picture of the properties of materials containing soluble but non-distillable components besides distillables, i.e. first hand information about potential processability of materials such as bitumens and "liquids" from coal liquefaction. The class analysis determines the amounts of oils, resins, asphaltenes and, in coal derived materials, preasphaltenes. The oils are distillable materials, mainly hydrocarbons, but also lower molecular weight, distillable heterocompounds containing sulfur, nitrogen or oxygen functionalities. The resins, asphaltenes and preasphaltenes are mixtures of compounds of high and progressively increasing molecular weight and are by definition "solubility classes." Preasphaltenes are materials soluble in pyridine or tetrahydrofuran but insoluble in toluene; asphaltenes are soluble in toluene, but insoluble in lower straight-chain hydrocarbons, and resins are materials which cannot be eluted with n-pentane from clays (Attapulgus, Fuller's), A standard method of class analysis, called SARA analysis (1), removes asphaltenes by n-pentane precipitation, while the solubles are applied on a clay column and separated into resins and oils. The oils can be further separated on silica gel or silica gel/alumina columns into saturates and aromatics of a progressively increasing number of rings. For coal liquids, the preasphaltenes are separated by toluene or benzene precipitation prior to the steps described above. The analysis is reliable and has a good reproducibility, but suffers from two main disadvantages. First, it is relatively slow (about 3-5 days are needed for its completion) and second, it is labor intensive and therefore not suitable for the analysis of larger numbers of samples. Attempts have been made to convert SARA analysis into a high-performance chromatographic method (2). Unfortunately HPLC is of limited value because available detectors are not easy to calibrate (UV/VIS, RI) and are difficult to operate (Pye moving wire FID). Another disadvantage of both gravimetric SARA analysis and its HPLC equivalents is due to what is often called "chemisorption," i.e. a certain part of the material tends to remain in the column and is extremely difficult to elute. Typically, recoveries are in the range of 82-95%. A third problem connected with column methods, mainly in classical SARA analysis, is the difficulty of removing residual solvent, because high-molecular weight compounds, e.g. preasphaltenes (PASP) and asphaltenes (ASP), retain residual solvent quite tenaciously.

We have, therefore, attempted to use TLC/FID for the determination of "class distribution" in materials such as high-boiling distillates (3), distillation residua (3) and the various extracts from coal liquefaction experiments (4), with the idea that if the FID response can be calibrated, the TLC of low volatile or non-volatile materials should eliminate all other disadvantages of column methods, i.e. remove the bottleneck of solvent removal and also provide direct accessibility to measurement of material which cannot be eluted from the column and thus escapes determination by column methods.

EXPERIMENTAL

For detailed experimental conditions, see References 3 and 4. The TLC/FID system consisted of an Iatroscan TH-10 instrument and a Hewlett-Packard 3390A integrator. All calculations are based on integrator counts. Rods,

Chromarod S-I; development systems, separation into hydrocarbons-resins-asphaltenes: 90% bed length with n-hexane (once or twice), 80% Bz/20% nC6 to 50% length, 40% methanol/methylene chloride to 30% length; separation into oils (maltenes)-asphaltenes-preasphaltenes, 1% methanol/nC6 to 90% length, 5% methanol/10% pyridine/benzene to 50% length. Each sample was analyzed 5 times in parallel and results averaged and std calculated.

RESULTS AND DISCUSSION

The development of the TLC/FID method for the various fossil liquids and extracts consisted of four main steps:

1. Development of a suitable separation system which would either reflect column separation or simulate separations based on solubility.
2. Establish method repeatability and reproducibility.
3. Establish concentration/detector response profiles.
4. Absolute calibration of detector response for the various compound classes.

The variety of materials encountered in fossil fuel chemistry precludes development of a single system capable of meeting all analytical demands. Transformation of a column separation into a TLC procedure does not present major difficulties. We expected that in addition to conventional type separations on silica gel it should be possible in principle to devise a separation system giving results closely resembling separations based on solubility only. The limited assortment of commercially available rods imposes limitations on the absolute comparability of solubility-based and chromatographic methods, since residual sorption phenomena always will be present in the latter. However, as Table 1 shows, the results correlate well.

We have described recently (3) separation of gas oil type materials and bitumens into oils, resins and asphaltenes and have shown that the correlation coefficients for linear regression of results obtained over a range of sample sizes were better than $r^2 = 0.98$, and that method reproducibility for this type of samples was better than that of column methods. An additional advantage of TLC using the Iatroscan instrument rests in the possibility to run in parallel up to 10 replicate analyses of a sample, thus increasing result precision. We have extended the work by devising separation systems capable of separating coal-derived extracts into preasphaltenes, asphaltenes and oils (4), by first developing the bed with 1% MeOH/nC6 to 90% of its length, to move the "oils," followed by a second development to 50% bed length with 5% MeOH/10% Py/Bz to displace asphaltenes from preasphaltenes with subsequent visiualization of the separated zones by FID. That this sequential development effects essentially "solubility"-based separation was verified by semi-preparative separation of the sample on TLC plates with subsequent extraction of the separated zones and infrared spectrometry to show that no discernible accumulation of functionalities took place (Fig. 1). The results were, of course, also compared with the results of separations based on selective solubility (precipitation) and rechromatographing of the individual preparatively separated fractions using TLC/FID (Fig. 2), showing the degree of cross-contamination of the preparatively separated fractions. The system used in our work should not be unique. Other solvent combinations are possible (5) using mixtures of n-pentane/iso-propanol and benzene/iso-propanol to achieve maltene, asphaltene and preasphaltene separation, provided that the systems are polar enough and that differential solubility for ASP and PASP is achieved. Again, method reproducibility was shown to be superior to results obtained by the classical gravimetric analysis, and the influence

TABLE 1

Comparison of TLC and SARA Analyses

	Aromatics+		Asphaltenes	
Sample	TLC	SARA[a]	TLC	SARA
R1	49.7	39.2	48.8	52.3
R14	35.4	32.8	64.2	64.8
R15	43.4	34.2	56.5	57.5
R24	83.1	73.9	16.8	22.1
R29	87.9	82.8	11.9	7.1
R18R	51.3	44.8	48.8	41.5

[a]Asphaltenes determined by precipitation with 40 vol n-hexane, etc.

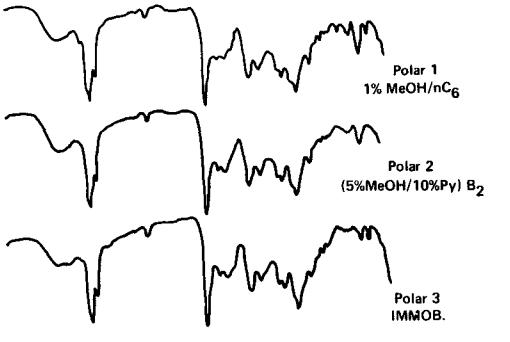

FIG. 1. IR spectra of fractions obtained from non-selective "solubility" separation by TLC.

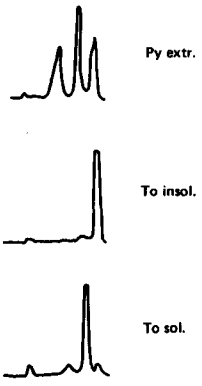

FIG. 2. TLC of fractions from solubility separation.

TABLE 2

Precision of TLC/FID Measurements for Actual Pyridine Extracts from Coal Liquefaction

Sample	Oils	s.d.	ASP	s.d.	PASP	s.d.
072	25.7	2.1	41.1	0.8	33.2	1.3
073	24.4	1.5	41.6	0.5	34.0	1.0
ANO-22-B	54.1	2.1	42.7	1.4	3.2	0.7
ANO-22-A	64.3	0.9	33.5	0.2	2.1	0.6
B-4-01	81.8	1.3	14.4	1.4	3.8	0.1
B-5-01	89.6	3.6	8.2	3.0	2.3	0.3
19-HV2	15.6	0.7	61.7	1.4	22.8	0.7
079	13.8	0.3	37.5	0.7	48.6	0.4

Each result is an average of 5 parallel determinations from which standard deviation is calculated.

Samples were chosen to cover wide ranges of relative contributions of the 3 classes of compounds.

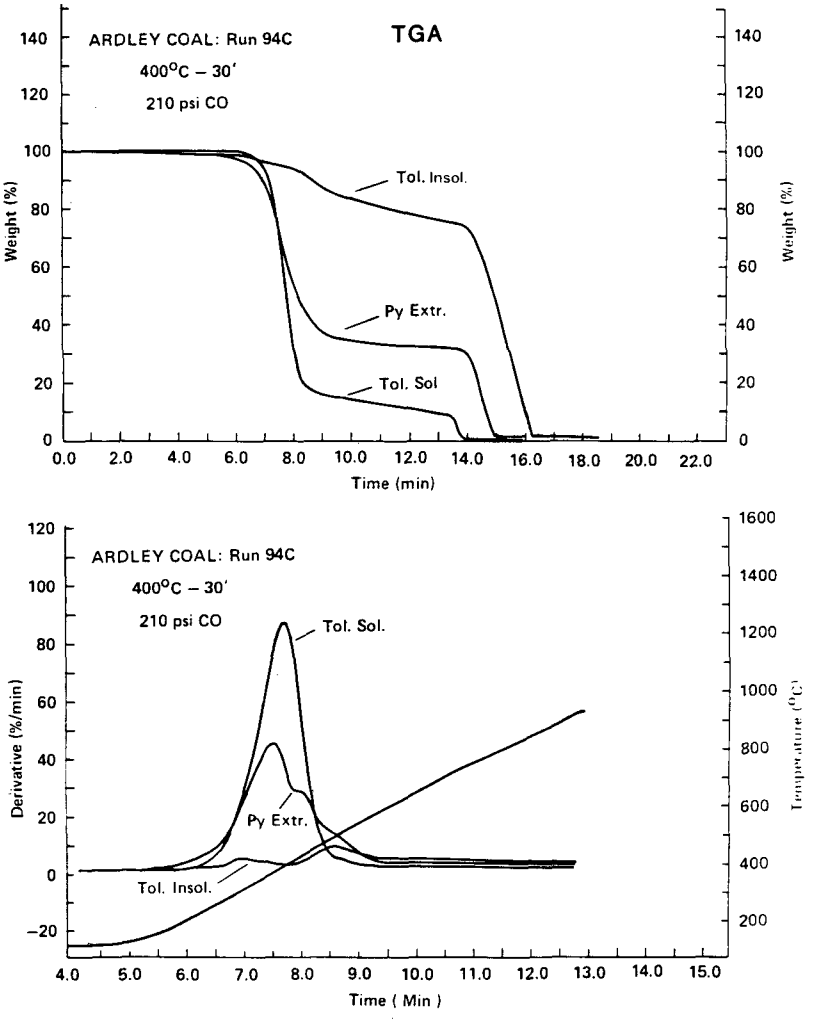

FIG. 3. Thermogravimetric analysis of separated fractions. The proportion of non-reactive carbon is shown.

of incomplete sample recoveries (amounting to 18% in some cases) also was eliminated. Table 2 summarizes results and standard deviations obtained for a series of samples substantially differing in their relative oil, asphaltene and preasphaltene contents.

Another type of backup analysis used was thermogravimetrically, which demonstrates the presence of non-distillable residua, thermally strippable carbon and non-reactive carbon beside the ash contents. TGA analysis has shown that the separated oils did not leave any residuum, and asphaltenes and preasphaltenes obtained by TLC separation behaved very much like fractions from separations by selective solubility (precipitation) (Fig. 3).

Once a suitable development has been established for these materials, the attention was focussed on the determination of response profiles, i.e. on the linearity or non-linearity of detector response for varying absolute amounts of the respective fractions. This is not a simple task in fossil fuel analysis, because the various components present in the various compound classes depend on each other for mutual solubility, and it often happens that a fraction eluted with a certain solvent will remain largely insoluble once the solvent is completely evaporated and sample redissolution in the same solvent attempted (6). To overcome this difficulty, the original extracts were used without solvent evaporation in such a way that the exact volume of extract was determined, sample applied for TLC/FID, then the sample solution was concentrated stepwise with successive TLC/FID analyses until, finally, the solvent was completely evaporated, residual sample weighed and the individual sample amounts calculated based on the weight of the residuum, as schematically shown in Figure 4.

Once detector response for absolute sample amounts was established (Fig. 5), simple statistics were used for response/concentration profile evaluation. Regression analysis was done simultaneously for the following functions: $y = a+bx$, $y = ax^b$, $y = ab^x$, $y = a + b/x$, $y = x/(a+bx)$, $y = a.\exp(bx)$, etc. Altogether, 10 different functions and regressions yielding the highest correlation coefficient were evaluated. In most cases, the function giving the largest r^2 was $y = a.x^b$, with both a and b close to one. Thus, an exponential response vs concentration seems to be the most general case. Since both a and b were close to one, it is obvious that this function can be replaced by a linear relationship over a limited sample amount range without substantially impairing the correlation coefficient. In practical terms, a suitable sample size proved to lie between 10-60 μg (Figs. 5 and 6).

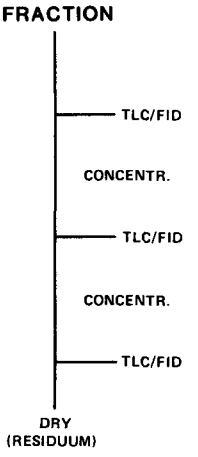

FIG. 4. Procedure for obtaining response/amount dependence curves.

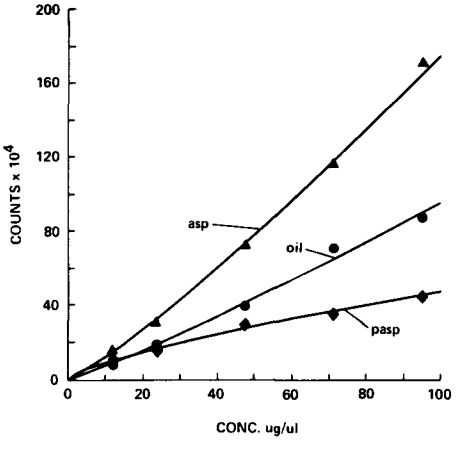

FIG. 5. Example of sample amount/response dependence for the separation of a sample into oils, ASP and PASP.

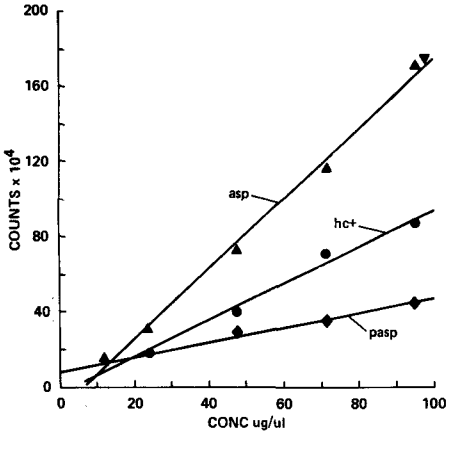

FIG. 6. Plot from Fig. 5 treated by linear regression.

In order to establish the relative FID response for the individual separated fractions, i.e. oils, asphaltenes and preasphaltenes, a procedure similar to that described earlier was used, with fractions obtained from semipreparative TLC (Figs. 7, 8 and 9) showing the adopted approach. First, the sample volume was adjusted to an accurate value and TLC/FID carried out. The sample was split further into a larger part which was evaporated to dryness, and the weight of residuum determined and sample concentration calculated. Another part of the sample was subjected to preparative TLC and the separated zones scraped and exhaustively extracted with the proper solvent(s). The volumes of the individual fraction solutions were adjusted to known values and TLC/FID carried out as described previously.

It is known that a carbon atom carrying a heteroatom such as N, O or S gives a lower relative response than a C bonded to other C or to H atoms. Due to the relatively large heteroatom contents in the asphaltenes and preasphaltenes, these two classes should give a somewhat lower relative response than the oils. It was shown that for the sample types tested, the response factors for asphaltenes and preasphaltenes were, respectively, 1.17 and 1.22, and that, while the response profile for oils was somewhat steeper, the asphaltene and preasphaltene response profiles ran parallel to each other (Fig. 10).

Unlike gas chromatography, where only sample vapors enter the detector, in TLC/FID dealing with non-volatile and sometimes poorly combustible materials, the rate of sample passage through the hot zone can prove to be an important factor. Testing of FID response at various scanning rates, followed by a second or even by several subsequent passages of the same rod through the detector flame, showed that oils did not give any response upon second passage and asphaltenes and preasphaltenes gave only a negligible response in the second passage (Fig. 11), provided that the rod was turned in the rack by 180° prior to FID scanning. The flame temperature was not sufficient to burn off solid particles, if present in the sample. Thus removal of solids from the sample prior to analysis is mandatory. Several hundred analyses of pyridine extracts from coal liquefaction experiments have revealed that the presence of solid particles is signalled by lower precision of measurements (typically standard deviation more than 3).

For routine work, composition dependent correction factors were assumed homogeneous and results of five parallel runs on a sample were averaged and standard deviations determined for each of the separated fractions. The

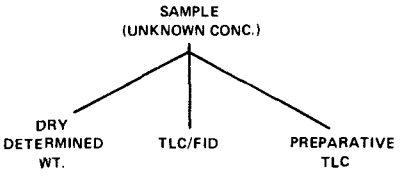

FIG. 7. Schematic of sample treatment for obtaining absolute detector calibration (preseparation step).

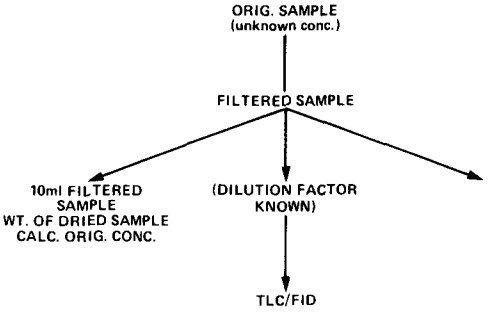

FIG. 8. Absolute detector calibration (determination of dilution factor).

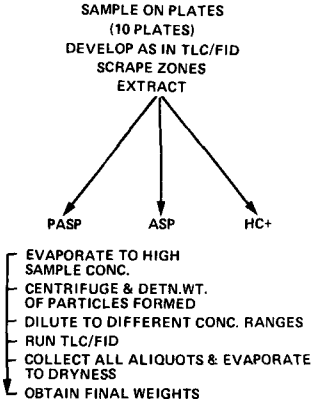

FIG. 9. Absolute detector calibration (determination of final weights of fractions).

TLC separation and FID measurements take less than two hours, and they are sufficiently fast for the treatment of large numbers of samples (even those available in microquantities only). The technique also was used successfully by Yokoyama et al. (7) for the separation of compound classes in coal hydrogenation liquids, and by us in the evaluation of products from small scale electrolytic reduction of petroleum and coal derived asphaltenes on Hg electrode (8). Result precision is better than that of column techniques. On the other hand, non-

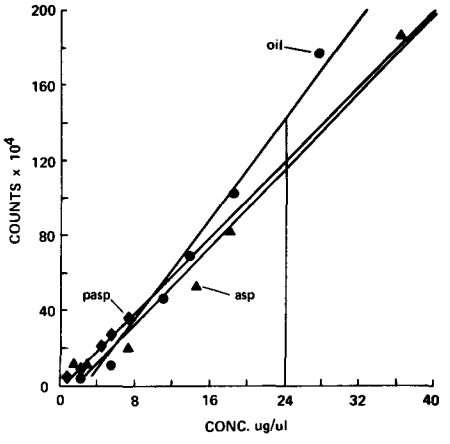

FIG. 10. Absolute calibration (plot of detector response vs equal fraction weight).

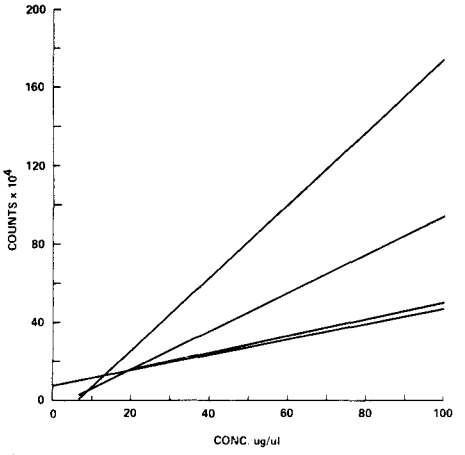

FIG. 11. Influence on the PASP signal of second passage of rod through the flame.

linearity of response over larger concentration ranges renders the use of internal or external standards for absolute measurements questionable (5) unless all of the above discussed variables are controlled within admissible limits.

ACKNOWLEDGMENTS

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Quantitative Determination of Lipids and Their Constituents by the Chromarod TLC-FID System

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ABSTRACT

The methanolysis products of neutral sphingolipid and archaebacterial neutral glycolipid were separated on Chromarods S-II (silica gel) with a double developing system. The lipid constituents separated on the rods were scanned automatically with a hydrogen flame ionization detector (Iatroscan). The molar ratios of the constituents determined by this system were very close to the theoretical values of the lipid.
Lipids 20:552-554, 1985.

INTRODUCTION

The usefulness of the Chromarod TLC-FID system for quantitative analysis of lipids already has been reported in several of our papers which discuss the use of this system in conjunction with the argentation and boric acid impregnated rod methods (1-4). Several reviews also have been published (5-7). This analytical system also has been applied to advantage in determining the relative amounts of nonpolar lipids, phospholipids and glycolipids in a mixture of bacterial lipid extract (8). In a previous paper, we described several basic experimental conditions for determining complex lipid constituents (9). In this paper, we discuss the applications of this system to the quantitative analysis of the constituent lipid and non-lipid moieties of sphingoglycolipids and archaebacterial lipids.

EXPERIMENTAL

Dihydroxysphingosine (Sph), methyl 12-hydroxystearate (h-$C_{18:0}$-Me), methyl lignocerate ($C_{24:0}$-Me), and ceramide monohexoside (CMH, from bovine brain) were purchased from Serdary Research Laboratories Inc. (London, Ontario, Canada) and methyl glucoside (Glc-Me) from Sigma Chemical Co. (London, U.K.). Methyl galactoside (Gal-Me) and glycerol were obtained from Wako (Osaka, Japan) and methyl stearate ($C_{18:0}$-Me) from Tokyo Kasei Kogyo (Tokyo, Japan). Archaebacterial lipids (GL-2, GL-3) were isolated from thermophilic acidophilic bacterium TA-1 (10-12).

The equipment used was an Iatroscan TH-10 analyzer (Iatron Lab. Inc., Tokyo) equipped with a Chromatogram Processor 7000 AS (System Instruments Co. Ltd., Tokyo). The Chromarods, type S-II (5 μm particle size silica gel), were used for separation of the lipid constituents. The lipids were methanolyzed with 5% methanolic-HCl and the whole methanolysate, without any further treatment, was analyzed to identify the lipid and non-lipid moieties. The methanolysates of ceramide monohexoside, sphingomyelin and archaebacterial lipids (GL-2, GL-3) were prepared by the following process. Five mg of each lipid sample was treated with 2 ml of 5% HCl-methanol (Kokusan Kagaku Co. Ltd., Tokyo) in a screw-cap tube at 100 C for 3 hr. After cooling, the solvent was evaporated and the residue dried over NaOH under vacuum. The residue was dissolved in chloroform-methanol (1:2). Chromarods were activated by passing them through the TLC-FID analyzer just before sample application. Activated rods were spotted with 1 μl of sample solution containing 5 mg/200 μl of a mixture of the methanolysates. The rods were developed with a solvent mixture at room temperature (about 20 C), dried at 120 C for 5 min and scanned with the Iatroscan analyzer.

RESULTS AND DISCUSSION

The standard mixture of methylglycosides, fatty acid methyl esters and sphingosines were resolved on Chromarod S-II by a double developing technique. The first development was made with a solvent mixture of chloroform-methanol-conc. ammonium hydroxide (60:10:1) for a distance of 6 cm from the origin. After being dried under vacuum for 10 min, the rods were developed again to a distance of 10 cm with the solvent mixture n-hexane-diethyl ether (50:20). A typical chromatographic separation of a mixture containing methyl glucoside, methyl galactoside, dihydroxysphingosine and methyl 12-hydroxystearate (h-$C_{18:0}$-Me) on a Chromarod S-II is shown in Figure 1. Methyl

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glycoside and sphingosine were separated with the first solvent mixture. The fatty acid methyl ester and the hydroxy fatty acid methyl ester were separated with the second solvent mixture.

The components separated on the Chromarod were automatically scanned with the Iatroscan hydrogen flame ionization detector. The relative responses of methyl glucoside and methyl galactoside to sphingosine, or to fatty acid methyl ester, were in the same proportion as the molar ratios. The relationship between the peak-area ratio (y) and the molar ratio (x) of the methyl glycosides (methyl galactoside and methyl glucoside) to the fatty acid methyl ester (methyl stearate) was found to be y = 0.2x (Fig. 2).

This system was applied to the analysis of the complex lipids of archaebacteria (*sulfolobus*).

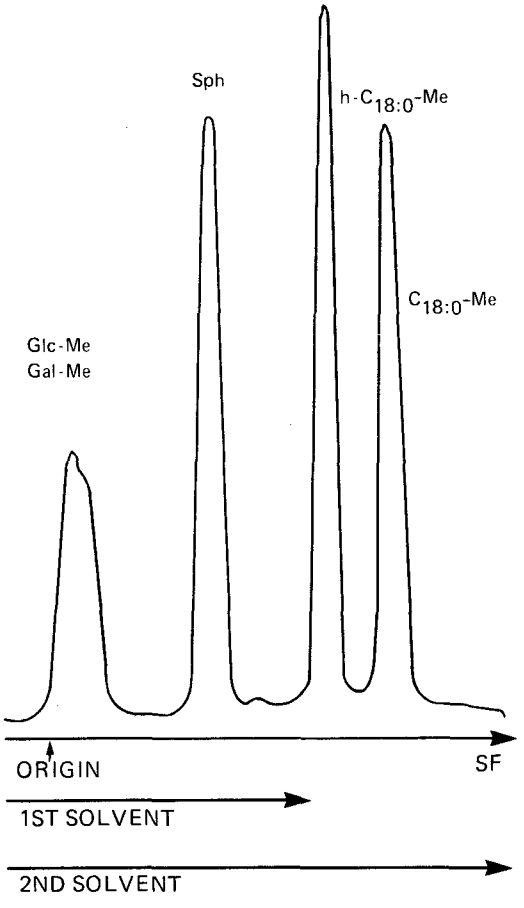

FIG. 1. Typical chromatographic separation of a mixture containing Glc-Me, Gal-Me, Sph, h-$C_{18:0}$-Me, and $C_{18:0}$-Me on a Chromarod S-II. First solvent, chloroform-methanol-conc. ammonium hydroxide (60:10:1); second solvent, n-hexane-diethyl ether (50:20). SF = solvent front.

The structure of the major neutral glycolipid (GL-2) of thermophilic acidophilic bacterium TA-1 is shown in Figure 3. GL-2 was converted to methyl glucoside, methyl galactoside and

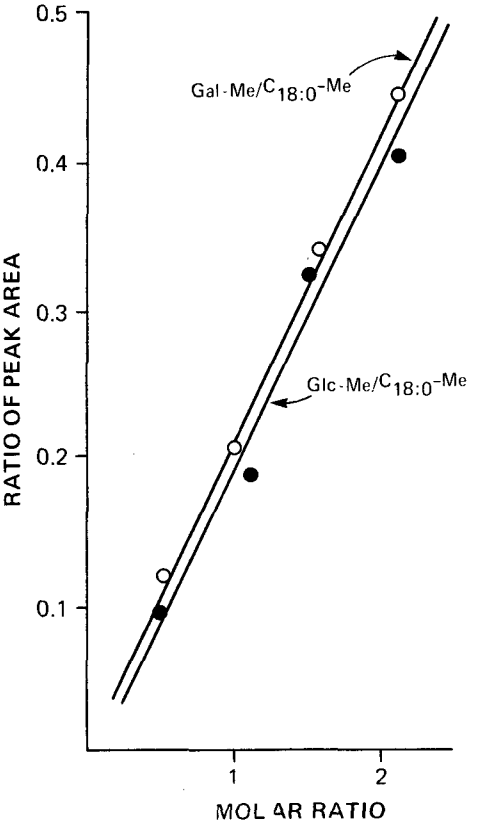

FIG. 2. Relationship of the peak-area ratio and molar ratio of methyl glycosides to fatty acid methyl ester.

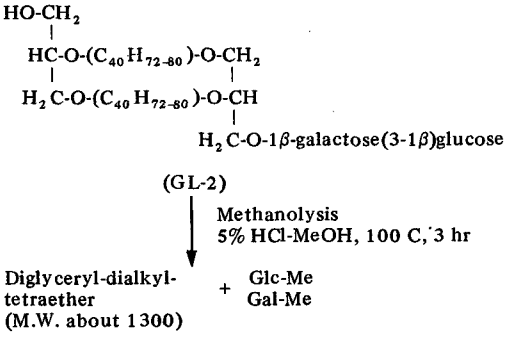

FIG. 3. Structure of GL-2 and its methanolysis products.

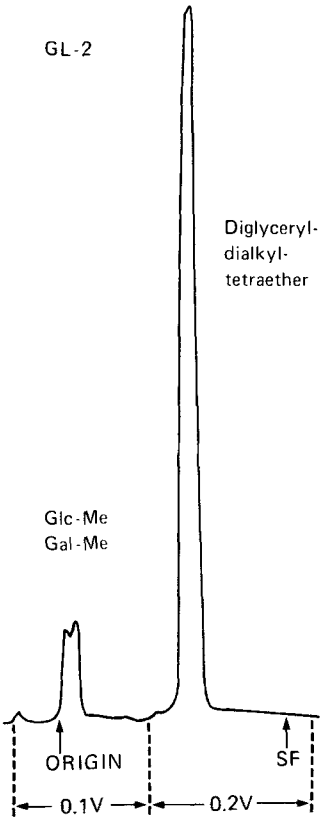

FIG. 4. Typical chromatographic separation of the methanolysates of a neutral glycolipid GL-2. The developing solvent was chloroform-diethyl ether (1:1).

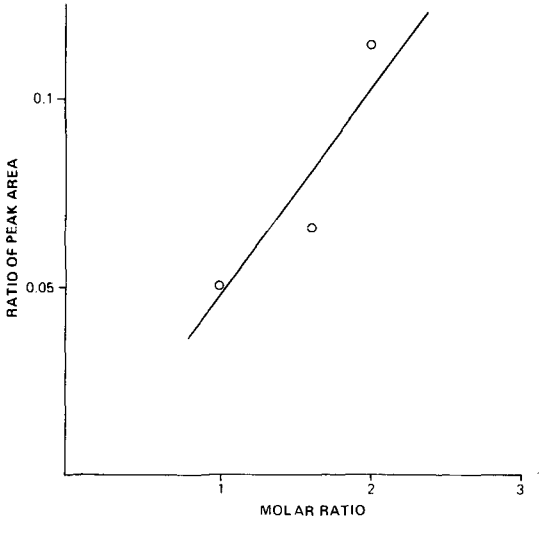

FIG. 5. Relationship of the peak-area ratio and molar ratio of methyl glucoside to diglyceryl-dialkyl-tetraether (DGTE).

TABLE 1

Comparison of the Peak-Area Ratio with the Molar Ratio of Neutral Glycolipid GL-2

	Hexose-Me/DGTE
Average peak-area ratio	0.09
Coefficient of variation (%)	7.5
Molar ratio	1.8

Hexose-Me: methyl hexoside; DGTE: diglyceryl-dialkyl-tetraether.

diglyceryl-dialkyl-tetraether (Fig. 3). A typical Chromarod separation of the methanolysates of GL-2 is shown in Figure 4. The relationship of the peak-area ratio (y) and molar ratio (x) of methyl hexosides (methyl galactoside and methyl glucoside) to diglyceryl-dialkyl-tetraether was found to be $y = 0.05x$ (Fig. 5). The molar ratio of methyl hexoside to diglyceryl-dialkyl-tetraether of GL-2 was calculated from the data in Figure 5.

The molar ratio of diglyceryl-dialkyl-tetraether to the methyl hexoside in the methanolyzate of the bacterial neutral glycolipid (GL-2) was 1:1.8 and very close to the theoretical value (Table 1). The reproducibility of the peak-area ratio was within 8% (C.V.).

Thus, the TLC-FID method is useful for determining relative amounts of complex lipid constituents.

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Quantitative Analyses of Methyl Esters of Fatty Acid Geometrical Isomers, and of Triglycerides Differing in Unsaturation, by the Iatroscan TLC/FID Technique Using $AgNO_3$ Impregnated Rods

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ABSTRACT

The relative FID responses for Iatroscan analyses of *cis* and *trans* isomers of methyl esters of 18:1Δ6, 18:1Δ9 and 18:1Δ11 on Chromarods-S impregnated with $AgNO_3$ were studied at load levels ranging from 0.5 to 20 μg, using methyl stearate as internal standard. The FID response correction factors were greater for the *cis* than for the *trans* isomers. The correction factors were relatively constant in the 10-20 μg interval, but increased in the range 0.5-5 μg.

Separation of tristearin, triolein, trilinolein and trilinolenin also was obtained on Chromarods-S impregnated with $AgNO_3$ using a mixture of benzene:chloroform:acetic acid (90:8:2) as the solvent system. The relative FID responses for the triolein, trilinolein and trilinolenin were determined at load levels ranging from 0.5 to 14.3 μg using tristearin as an internal standard. The FID response correction factors of these three triglycerides differed significantly for load levels of 1.0, 2.5 and 5.0 μg. However, the factors could be considered as being equal in the range 10 to 14.3 μg.

Correction factors were not affected by repeated re-use of the same set of Chromarods. Several hundred separations and scans appeared feasible.

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INTRODUCTION

Thin-layer chromatography (TLC) on a silica gel-glass frit coating on a quartz rod (Chromarods-S), with scanning by a flame ionization detector (FID), has been in use for only a few years (1). In addition to direct analyses of organic compounds, such as lipids of all types, the use of rods impregnated with $AgNO_3$ has been applied to the separation of methyl esters of fatty acids with different degrees of unsaturation (2) (saturates, monoenes, trienes), as well as of the triglycerides combining these acids in different degrees of unsaturation (3,4). This method also was applied to the quantitation of esters of *trans* fatty acids in partially hydrogenated vegetable or marine oils (2,5,6). A large amount of data is available on the FID response correction factors of different lipid classes when analyzed by untreated Chromarods (7-9). However, relatively little is available on the FID response correction factors for unsaturated geometrical fatty acid isomers and triglycerides when analyzed on $AgNO_3$-Chromarods (2,6). Therefore, we have studied the response correction factors of *cis* and *trans* 18:1 isomers and unsaturated triglycerides (triolein, trilinolein and trilinolenin) as a function of the amount of sample spotted and the age of the Chromarods.

EXPERIMENTAL PROCEDURES

All solvents were redistilled before use. The methyl ester standards *cis* and *trans* 18:1Δ6, 18:1Δ9 and 18:1Δ11 and the triacylglycerols, tristearin, triolein, trilinolein and trilinolenin were purchased from Nu-Chek-Prep, Inc., Elysian, Minnesota).

Silver nitrate thin layer chromatography of geometrical fatty acid isomers on Chromarods-S. Clean rods were immersed in a 2.5% solution of silver nitrate in acetonitrile for 15 min, then oven-activated at 120 C for 2-3 hr. These rods were spotted with the appropriate amount of sample (0.5 to 20 μg) and of internal standard (18:0, 10 μg) and developed for 25 min in a mixture of hexane/benzene (1:1). The rods were then scanned (0.24 cm/sec) by the FID in an Iatroscan TH-10 apparatus (160 ml/min of hydrogen and 2000 ml/min of air). A 1 mV Linear Instruments recorder was used to record the output. Peak areas were measured on a Technicon (Chauncey, New York) Model AAG integrator/calculator. After use, the rods were cleaned for re-use by soaking in concentrated nitric acid for several hours, then rinsing with water and acetone, and finally by a complete burn through the flame.

Silver nitrate thin layer chromatography of

triglycerides on Chromarods-S. The same procedure as described above for methyl esters was used to study the response correction factors of unsaturated triglycerides. The rods were developed for 30 min in a mixture of benzene: chloroform:acetic acid (90:8:2). Tristearin was used as an internal standard with 10 μg spotted on each rod.

RESULTS AND DISCUSSION

FID responses of some fatty acid methyl esters. Fatty acid methyl esters differing in degree of unsaturation and in geometry were separated readily on Chromarods-S coated with $AgNO_3$ using a mixture of benzene:hexane (1:1) as the solvent system (2). However, this solvent system did not permit the separation of *cis* or *trans* monounsaturated fatty acids which differed in the position of the ethylenic bond on the carbon chain. The separation of positional *cis* or *trans* isomers on TLC also was found to be minimal for centrally-located bonds compared to those at the ends of the chains (10).

A very good correlation was found in the range of 0.5 to 20 μg between the amount of unsaturated methyl esters spotted on the rods and areas obtained relative to the internal standard (18:0). For example, for 18:1Δ6t (Fig. 1), the linear regression (0.5 to 20 μg) gave a correlation coefficient of 0.991. The resulting response regression line did not pass through the origin (Fig. 1). Two separate regressions, one from 0.5 to 5 μg and one from 5 to 20 μg, produced a slightly better fit than a single regression (Fig. 1). The regression of the lower points would pass very close to the origin, while that for the upper points would not. The cross-over point for these two regressions would be between 3 and 5 μg. The results for all the *cis* and *trans* methyl esters also can be expressed as non-linear regressions (Table 1). In this case, a polynomial regression gave only a slightly better fit than a linear regression, and any of these equations (Table 1) could well describe the behavior of monounsaturated methyl esters when analyzed by the TLC-FID system.

Similar curvilinear equations were found by Phillips et al. (11) for the quantitative analyses of lipid classes using high performance liquid chromatography (HPLC) with a flame ionization detector. This is also in agreement with data presented by Delmas et al. (12) on the analysis of marine lipids by the TLC/FID system.

The response correction factors of the three *cis* and *trans* 18:1 isomers were calculated from the response calibration curves based on the internal standard (18:0) having a correction factor of 1.00. For both the *cis* and *trans* 18:1 isomers, the correction factors decreased with an increase in the amount of sample spotted (Fig. 2). The major changes occurred in the 0.5-5 μg interval. However, in the 10-20 μg interval, the correction factors were fairly constant.

Analysis of variance (13) showed that for a sample load ranging from 5 to 20 μg, both the geometry and the position of the ethylenic bond affected the FID correction factors of

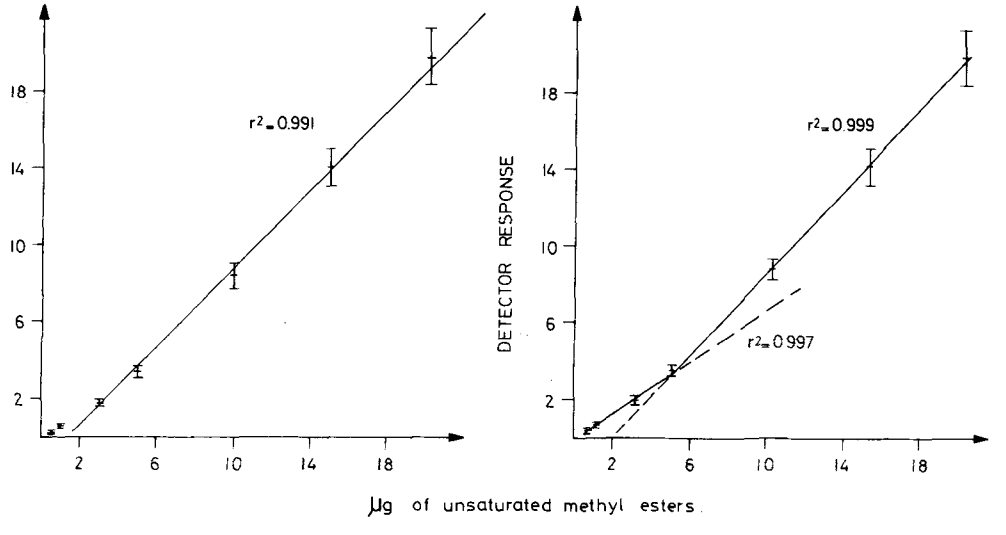

FIG. 1. Plot of relative detector response vs the amount of *trans* 18:1Δ6 applied on $AgNO_3$-Chromarods. Internal standard, 10 μg of 18:0. Each point is the mean of 10 rods. Left, one regression line using all points; right, two regression lines; 0.5 to 5 μg, and 5 to 20 μg.

TABLE 1

Calibration Curve Regression Equations for *cis* and *trans* Monounsaturated Methyl Esters

Compound	Equations	Correlation (r^2)
*trans*Δ6	$0.0524 + 0.4660x + 0.0471x^2 - 0.0011x^3$	0.994
	$-0.2811 + 0.7254x + 0.0136x^2$	0.993
	$-0.9555 + 0.9987x$	0.991
*trans*Δ9	$-0.4702 + 0.8945x + 0.0115x^2$	0.995
	$-1.1808 + 1.1367x$	0.994
*trans*Δ11	$-0.0786 + 0.0609x + 0.0363x^2$	0.995
	$-1.0043 + 1.1653x$	0.988
*cis*Δ6	$0.1060 + 0.4300x + 0.0436x^2 - 0.0014x^3$	0.985
	$0.2720 + 0.7400x + 0.0023x^2$	0.983
	$-0.3760 + 0.7850x$	0.983
*cis*Δ9	$-0.2620 + 0.7630x + 0.0053x^2$	0.985
	$-0.5970 + 0.8750x$	0.984
*cis*Δ11	$-0.0510 + 0.5555x + 0.0321x^2 - 0.0007x^3$	0.996
	$-0.1552 + 0.6694x + 0.0137x^2$	0.995
	$-0.5748 + 0.9043x$	0.993

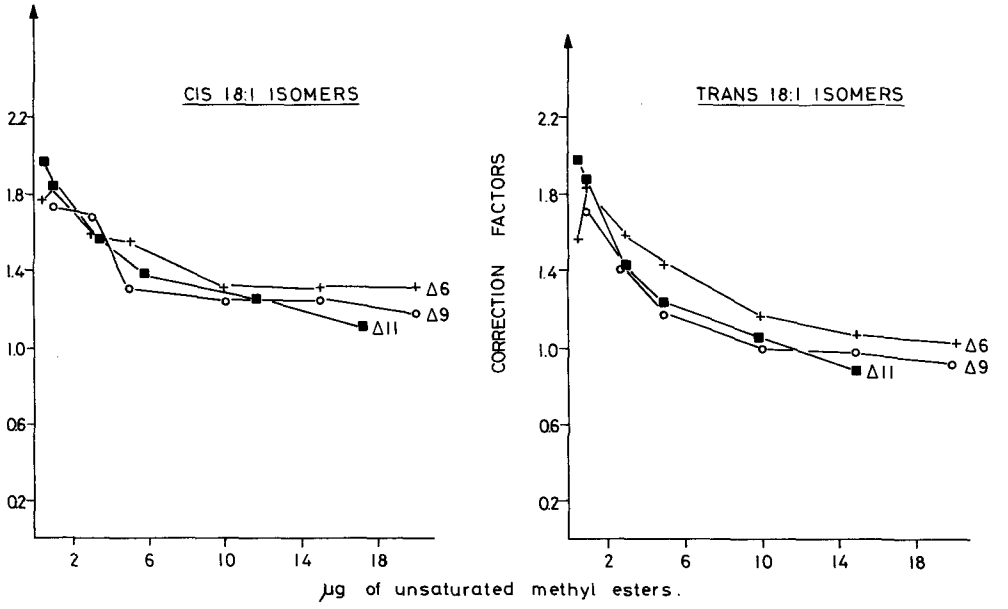

FIG. 2. Relative response correction factors for *cis* and *trans* 18:1 isomers when analyzed by the $AgNO_3$-TLC/FID system. Internal standard, 10 μg of 18:0.

unsaturated fatty acids (Fig. 2). The correction factors for the *cis* isomers were higher than those for the *trans* isomers. Duncan's test (13) showed that between 0.5 and 3 μg the geometry of the ethylenic bond did not influence the correction factors. The TLC/FID technique on $AgNO_3$ rods is a complex system. One has to consider the interaction between the silver ion and the ethylenic bond. *Cis* isomers are more strongly absorbed than the corresponding *trans* isomers, and less complete or delayed liberation from the complex could lead to a larger correction factor for the former geometric isomer.

FID responses of some triglycerides. The calibration curves for triolein, trilinolein and trilinolenin were obtained using tristearin as an internal standard. These curves were similar to those obtained for the unsaturated methyl esters. Two types of linear regressions could be calculated (Fig. 3). For example, two separate regression lines for triolein, one from 0.5 to 5 μg and one from 5 to 14.3 μg, gave excellent fits (r^2 =

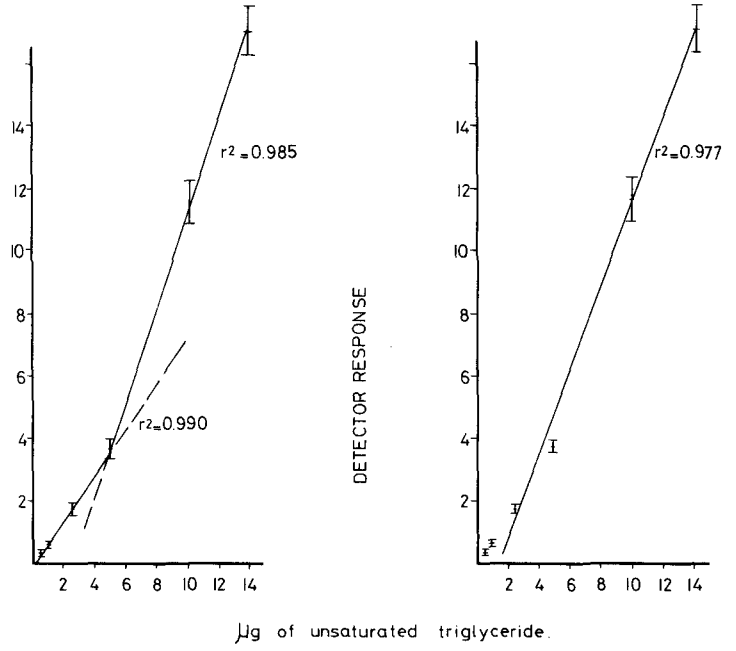

FIG. 3. Plot of relative detector response vs the amount of triolein applied on $AgNO_3$-Chromarods. Internal standard, 10 μg of tristearin. Each point is the mean of 10 rods. Left, two regression lines; 0.5 to 5 μg and 5 to 14.3 μg. Right, one regression line using all points.

TABLE 2

Calibration Curve Regression Equations for Unsaturated Triglycerides

Compound	Equations	Correlation (r^2)
Triolein	$0.3882 - 0.0372\,x + 0.1871\,x^2 - 0.0072\,x^3$	0.989
	$-0.4212 + 0.8320\,x - 0.0286\,x^2$	0.983
	$-1.1070 + 1.2460\,x$	0.977
Trilinolein	$0.4172 + 0.1837\,x + 0.1932\,x^2 - 0.0078\,x^3$	0.983
	$-0.4537 + 1.1200\,x + 0.0225\,x^2$	0.979
	$-0.9924 + 1.4450\,x$	0.976
Trilinolenin	$-0.0001 + 0.8243\,x + 0.1230\,x^2 - 0.0056\,x^3$	0.985
	$-0.5305 + 1.4520\,x + 0.0026\,x^2$	0.984
	$-0.5922 + 1.4890\,x$	0.984

0.985 and 0.990), whereas one linear regression line from 0.5 to 14.3 μg gave a correlation of 0.997. However, this regression line (Fig. 3) did not pass through the origin. Similar results were obtained for trilinolein and trilinolenin. The cross-over point for the two regression lines would be between 4 and 5 μg. In agreement with data from unsaturated methyl esters, curvilinear equations could be calculated (Table 2). In general, a polynomial regression gave a better fit than a linear regression except for trilinolenin, where the three different fits were equally good.

Analysis of variance (13) showed that for each triglyceride, as with the methyl esters, the FID response correction factors decreased significantly as the amount of sample spotted on the rods increased (Table 3). The Newman-Keul test (13) indicated that the FID response correction factors of the three triglycerides were significantly different for load levels of 1.0, 2.5 and 5.0 μg. These factors could be considered as being equal in the range 10 to 14.3 μg. A decrease in response correction factors with sample quantity has been reported recently for both

TABLE 3

Influence of Amount Spotted on FID Response Correction Factors of Unsaturated Triglycerides When Analyzed on $AgNO_3$-Chromarods

Amount spotted (μg)	Triolein	Trilinolein	Trilinolenin
0.5	1.87 ± 0.31[a]	1.33 ± 0.19	1.37 ± 0.19
1.0	1.79 ± 0.22	1.39 ± 0.15	1.17 ± 0.17
2.5	1.46 ± 0.19	1.00 ± 0.10	0.79 ± 0.09
5.0	1.37 ± 0.11	1.07 ± 0.12	0.80 ± 0.08
10.0	0.86 ± 0.04	0.72 ± 0.06	0.68 ± 0.05
14.3	0.85 ± 0.08	0.73 ± 0.08	0.70 ± 0.07

[a]Average of 10 rods ± standard deviation.

TABLE 4

Influence of Age of Rods on FID Response Correction Factors for *cis* and *trans* 18:1Δ9 (10 μg spotted per rod)

Times rods used	Correction factors	
	cis[a]	*trans*[a]
14	1.04	0.97
20	1.15	0.87
32	1.08	0.94
37	1.09	0.93
44	1.07	0.95
51	1.07	0.95
58	1.05	0.96

[a]Average of 10 rods; 10 μg of 18:0 used as internal standard; average precision of 10%.

triglycerides and methyl esters (14). Our study on $AgNO_3$ rods shows an increase in response correction factors only at low load levels.

CONCLUSION

An important feature of this $AgNO_3$-TLC/FID system is the longevity of the Chromarods-S. Rods easily can be re-used after cleaning in a concentrated nitric acid solution. The correction factors were not modified by the age of the rods (Table 4). This particular set of rods was, in fact, used for over 400 FID scans.

All of the data presented in this work were collected from consecutive scans on a set of 10 Chromarods. This use of different Chromarods is a generally accepted method of calibrating for lipids. It also has been demonstrated that the use of each rod as its own individual analytical unit gives better precision (12). However, the use of an internal standard should eliminate much of the variability that results from differences between rods (7).

The silver nitrate impregnated Chromarod FID technique has been used to study the isomer composition of mono- and diethylenic fatty acids in a given chain length of partially hydrogenated marine oils. For example, the diethylenic isomers of a partially hydrogenated menhaden oil (2,15) were fractionated into three peaks of increasing Rf, with benzene as developing solvent. The *cis,cis*-non-methylene-interrupted-dienes (NMID) migrated the least, then the *cis,cis*-methylene-interrupted-dienes (MID) mixed with the *cis,trans* + *trans,cis* NMID. The *trans,trans* NMID together with the *cis,trans* + *trans,cis* MID were the most mobile classes found.

One of the major applications of the $AgNO_3$-TLC/FID system is the quantification of *trans* fatty acids in margarines and in partially hydrogenated oils (2,6,16). In order to obtain an accurate quantification of the sample, the internal standard must be present at the same load for all calibrations, as well as for all sample analyses. Otherwise, the peak area ratios are likely to be different. More reproducible results will be obtained in the range 10 to 15 μg.

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Oral Polyunsaturated Phosphatidylcholine Reduces Platelet Lipid and Cholesterol Contents in Healthy Volunteers

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ABSTRACT

The effects of orally administered polyunsaturated phosphatidylcholine (PPC) on plasma lipids, lipoproteins and platelet function and composition were studied in seven healthy male volunteers. PPC (Nattermann & Cie, GmbH, Cologne, Federal Republic of Germany), 10 g/day, was given for a 6-week period after a 4-week wash out; laboratory tests were repeated after a further 4-week period after the end of treatment. PPC did not appear, during treatment, to modify the levels of plasma total cholesterol and triglycerides. High density lipoprotein (HDL) cholesterol levels were, however, increased after six weeks of PPC. The most dramatic changes occurred in platelet membrane composition: the total lipid/total protein and the cholesterol/protein ratios were reduced significantly, whereas increases of the phospholipid/total lipid ratio and of the linoleic acid membrane content were observed. Platelet function tests, both in whole blood and in platelet rich plasma, were not modified. Similarly, the thromboxane B_2 formation after standard stimuli and the sensitivity to exogenous prostaglandin I_2 also were unchanged. During the final wash out period following treatment, a reduction of plasma total and low density lipoprotein (LDL) cholesterol levels also was recorded. PPC appears to be capable of modulating lipid exchanges between cell membranes and the plasma compartment.
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INTRODUCTION

The effects of the administration of polyunsaturated lecithin (phosphatidylcholine, PC) from soybean have been investigated extensively in man in terms of the effects on plasma lipoproteins and, possibly, on central nervous system function (1). PC also has been reported to exert an antiatherosclerotic activity in different animal models (2,3). Following oral administration, PC is absorbed more than 90% by the intestinal mucosa, via conversion to the lyso product and reesterification (4,5). The absorbed PC is then incorporated into chylomicrons (5) and, after degradation of the triglyceride (TG) rich particles, taken up by the high density lipoprotein (HDL) fraction (4).

Little information is available on the subsequent fate of PC in relation to the possible incorporation into tissues and circulating cells in humans, although it has been shown that after administration of labelled PC labelling of erythrocytes occurs (4). The incorporation of PC into membranes may be of relevance for cells, such as blood platelets, where active phospholipid metabolism may be associated with modifications of the functional state (6).

The objective of this study, in human subjects, was to evaluate the effects of a prolonged (6 weeks) administration of a large amount of highly purified polyunsaturated phosphatidylcholine (PPC) from soybean on the composition of plasma lipoproteins and particularly of platelet membranes. In addition, the pattern of platelet aggregation and of platelet thromboxane formation was investigated in detail. The reported data suggest that significant changes in the platelet membrane composition may follow prolonged treatment with high oral doses of PPC.

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METHODS

Subjects

Seven healthy male volunteers, age 25.7 ± 3.1 yr and without clinical signs of disease, were selected for the study. The subjects did not take any drug affecting plasma lipids and platelet function for at least one mo prior to this study.

Drug Administration and Treatment Schedule

Purified PPC was prepared from Nattermann (Cologne, Federal Republic of Germany). Specified weight percentage composition was: 3-SN-phosphatidylcholine 68, medium chain triglyceride 12, fructose 6.5, orange flavor 3. The linoleic acid content of phosphatidylcholine was 72% of total fatty acids.

The product was provided as a paste, to be consumed with the morning breakfast. The daily PPC dose was 10 g. Treatment schedule consisted of a 6-week period of drug administra-

tion, preceded and followed by 4-week periods off PPC treatment. Blood samples were obtained in the following sequence: (weeks): four weeks before (-4), and at start of treatment (0), three weeks after 0 (+3), end of treatment (+6) and 10 weeks after treatment began (+10). Blood was drawn from the anticubital vein after an overnight fast, while the subjects were in a horizontal position, and without application of a tourniquet. Blood was drawn in Na citrate (3.8 g%, 1:10) as an anticoagulant. Studies of platelet aggregation and thromboxane B_2 (TxB_2) formation were carried out at -4, 0, +6 and +10 weeks. Platelet sensitivity to exogenous prostacyclin (PGI_2), cAMP levels in platelets after incubation with PGI_2 and platelet lipid composition, as well as lipoprotein levels as separated by preparative ultracentrifuge, were monitored at 0, +6 and +10 weeks.

Platelet Parameters

Whole blood aggregation. This parameter was evaluated in a whole blood aggregometer (Chrono Log Corporation, Havertown, California) by measuring the amplitudes of the aggregation curves obtained after the addition of increasing concentrations (2, 4, 8 μg/ml) of collagen (Hormon Chemie, Munchen, Federal Republic of Germany), 8 min after stimulation.

Platelet rich plasma (PRP) preparation. PRP was prepared from anticoagulated blood by centrifugation at 150 g and diluted with autologous PPP to the platelet concentration of 250×10^3 platelets/μl PRP.

PRP aggregation. Aggregation was evaluated in an aggregometer (Elvi Logos, Milan, Italy) by measuring the minimal concentrations of each aggregant, i.e. collagen and adrenaline (ISM, Milan, Italy) inducing aggregation curves with a minimum 50% decrease in optical density (threshold aggregating concentration, TAC), as well as the amplitude of the aggregation curves at a given concentration of each aggregant expressed in cm five min after the addition of the aggregating agent.

Thromboxane B_2 (TxB_2) formation in stimulated PRP-TxB_2. Formation was evaluated by measuring TxB_2 levels in PRP, two min after stimulation with 10 μg/ml of collagen. The reaction was stopped by the addition of 25 vol methanol. After evaporation of the solvent, samples were redissolved in the appropriate buffer and TxB_2 was measured by a specific RIA (7).

Sensitivity of PRP to exogenous prostacyclin (PGI_2). The concentrations of PGI_2 (Farmitalia Carlo Erba, Milan, Italy) inducing a 50% inhibition (IC_{50}) of the aggregatory response to collagen-induced aggregation (4 μg/ml) were determined.

Cyclic AMP levels after PGI_2 stimulation. The levels of cAMP accumulated in PRP were determined one min after incubation with 0.8×10^{-8}M PGI_2. At the end of the incubation the samples were frozen, thawed and subsequently acidified at pH 3.0 with 0.4 $NHClO_4$. The supernatants from high speed centrifugation were neutralized with 2 N Tris buffer and cAMP levels determined by a specific RIA (8), after separation on alumina and Dowex columns (9).

Composition of platelet membranes. 50 ml samples of collected blood were processed for the preparation of platelet membranes, by centrifugation procedures. One-fifth of the final platelet suspension in buffer was used for protein determination and the remainder for lipid analysis. The two platelet samples were further centrifuged and subjected to osmotic shock in distilled water, and the final pellets used for analytical determinations.

Proteins were determined by the Lowry method (10), and lipid analysis was carried out on chloroform-methanol 2/1 extracts (11). Total lipids were measured by weighing aliquots of the extract on a Cahn Gram microbalance after solvent evaporation. Total cholesterol in the lipid extract was determined by quantitative gas liquid chromatography (GLC) using stigmasterol as the internal standard. Total phospholipids (PL) were determined by phosphorus (P) determination on the lipid extract (12). Analysis of the fatty acid composition of total PL from platelet membranes was carried out by GLC of methyl esters on an SP-2330 column (Supelco Inc., Bellefonte, Pennsylvania), with programming temperature (150-210 C, 2.5 C/min increment). PL were isolated by one dimensional TLC (hexane/diethyl ether/acetic acid, 70:30:1, v/v/v as developing solvent) as the band remaining at the origin.

Lipid and Lipoprotein Determination

Plasma total cholesterol (TC) and triglyceride (TG) levels were determined by enzyme methodologies at -4 C, 0, +3, +6 and +10 weeks time intervals (13,14). Accuracy and precision of the methods were monitored continuously by the WHO Lipid Control Center in Prague; similarly, at each interval, high density lipoprotein (HDL) cholesterol levels were evaluated by a selective precipitation method (15). The complete lipoprotein fractionation was carried out by preparative ultracentrifuge, using standard density cuts (VLDL d < 1.006, LDL d < 1.006-1.063, HDL d < 1.063-1.210) (16). On each fraction, plasma TC and TG levels were determined.

Evaluation of statistically significant differences was carried out by paired t-test analysis of data for each individual at the various time intervals.

Plasma glucose was determined by a glucose-oxidase method (Kit Clinicals, Carlo Erba, Milan, Italy).

RESULTS

Clinical Observations

Administration of the PPC product was well tolerated by the volunteers, who complained only about the stickiness of the preparation, particularly in the early phases of treatment. Neither body weight nor blood pressure showed any significant change before, during or after PPC treatment.

Serum Lipid and Glucose Levels

No significant change in TC and TG levels was observed during treatment with PPC (Table 1). HCL-C levels, however, were increased at the end of PPC treatment. After the wash out period, total and LDL cholesterol levels were markedly reduced in respect of -4, 0 and +6 time periods. Serum glucose levels were significantly reduced during PPC treatment, and this reduction was reversed after the end of treatment.

Composition of Platelet Membranes

Platelet membrane composition was markedly modified after 42 days of PPC treatment (Table 2). Total lipid/total protein was significantly reduced. In the lipid fraction, the phospholipid content expressed as percent of total lipids was significantly increased and the resulting cholesterol/phospholipid ratio reduced. The percentage of cholesterol with respect to total lipids was unchanged, but the amount of cholesterol per mg protein was reduced more than 25%.

After the 4-week wash out the reduction in the total lipid/protein ratio and in the cholesterol/protein ratio were not significantly different from the values measured at the end of the treatment. In the lipid fraction, cholesterol increased as the percent of total lipids and also as related to phospholipids (C/PL ratio).

The analyses of the fatty acid composition of phospholipids from platelet membranes (Table 3) showed that about 50% of the total are saturated and that 18:2 and 20:4 account for about 20% of total fatty acids. PPC treatment significantly increased the linoleic acid level, whereas no change in the content of the other fatty acids was observed. Concomitantly,

TABLE 1

Plasma Lipid and Lipoprotein Levels (mg/dl) at the Various Time Intervals

Weeks	-4	0	+3	+6 (end of treatment)	+10 (end wash out)
Total cholesterol	232 ± 12[b]	230 ± 13[e]	220 ± 24	232 ± 17[d]	208 ± 13[b,d,e]
Total triglyceride	85 ± 8	91 ± 13	112 ± 23	106 ± 16	95 ± 12
LDL-triglyceride	50 ± 5	56 ± 11	70 ± 15	63 ± 10	64 ± 16
VLDL-cholesterol	12.3 ± 1.4	14.7 ± 2.7	15.7 ± 4.1	11.6 ± 1.8	16.2 ± 3.8
LDL-cholesterol	173 ± 13[e]	167 ± 11[e']	167 ± 15[a]	168 ± 13[d]	145 ± 12[d,e,e',a]
HDL-cholesterol	47.1 ± 2.3	47.6 ± 2.2[e]	48.3 ± 2.6	49.0 ± 2.4[e]	49.7 ± 3.6
LDL/HDL cholesterol ratio	3.71 ± 0.23[a,b,e]	3.53 ± 0.22[a]	3.52 ± 0.35[b']	3.46 ± 0.28[b,c,b']	2.96 ± 0.23[c,e]
Glucose	85.4 ± 1.5[c,d]	90.0 ± 2.1[b,c,d',e]	76 ± 3.0[a,b,b']	75 ± 1.6[d,e,e']	84.1 ± 2.5[a,d',e']

Data are the average ± SEM of values obtained in duplicate analyses for each volunteer at each time period. Values with the same superscript are significantly different from each other at the following levels: [a] $p: < 0.05$; [b,b'] $p: < 0.02$; [c] $p: < 0.01$; [d,d'] $p: < 0.005$; [e,e'] $p: < 0.001$.

TABLE 2

Platelet Protein and Lipid Composition at the Various Time Periods

Weeks	-0	+6 (end of treatment)	+10 (end wash out)
TL/P	0.287 ± 0.016[c]	0.239 ± 0.011[c]	0.255 ± 0.013
C as % TL	17.9 ± 0.66	16.1 ± 0.48[b]	17.2 ± 0.55[b]
PL as % TL	72.5 ± 1.44[a,b]	77.0 ± 1.64[a]	75.2 ± 0.79[b]
C/PL	0.247 ± 0.009[b]	0.210 ± 0.007[b,c]	0.231 ± 0.005[c]
μg C/mg P	51.4 ± 3.7[d]	38.4 ± 1.2[d]	44.1 ± 3.2

Data are the average ± SEM for values obtained in duplicate analyses for each volunteer at each time period.

TL, total lipid; P, protein; C, cholesterol; PL, phospholipids.

Values sharing common superscript are significantly different from each other at the following levels; [a]: $p < 0.05$; [b]: $p < 0.02$; [c]: $p < 0.01$; [d]: $p < 0.005$.

TABLE 3

Fatty Acid Composition (wt%) of Platelet Total Phospholipids

Fatty acids	0	+6 (end of treatment)	+10 (end wash out)
16:0	24.9 ± 1.1	25.0 ± 0.7	25.9 ± 1.5
18:0	24.0 ± 0.8	24.8 ± 0.5	24.7 ± 0.7
18:1	20.0 ± 1.1	18.4 ± 0.9	19.3 ± 0.8
18:2	4.4 ± 0.15[a]	5.1 ± 0.26[a,b]	4.4 ± 0.34[b]
20:3	5.2 ± 0.24	5.2 ± 0.17	4.7 ± 0.48
20:4	16.4 ± 1.2	16.8 ± 1.1	16.5 ± 0.7
24:0	1.8 ± 0.10	1.8 ± 0.14	1.6 ± 0.21
24:1	2.9 ± 0.16	2.9 ± 0.19	2.7 ± 0.31
Saturates	50.7	51.6	52.2
Monounsaturates	22.9	21.3	22.0
Polyunsaturates	26.0	27.1	25.6
20:4/18:2	3.73	3.29	3.75
20:4:20:3	3.15	2.23	3.51

Values (mean ± SEM) sharing common superscript are significantly different from each other at the following levels: [a]: $p < 0.01$; [b]: $p < 0.05$.

the 20:4/18:2 ratio tended to decrease after PPC treatment.

After the 4-week wash out period, the linoleic acid content of platelet phospholipids tended to return to basal values. In fact, the 18:2 content was different from that after PPC treatment although less significantly so ($p < 0.05$).

Platelet Parameters

Aggregations. The amplitudes of whole blood aggregation waves induced by increasing concentrations of collagen were measured before and after oral PPC treatment. No difference, at any time interval or at any collagen concentration, was statistically significant. The data obtained with 1 μg/ml collagen are shown in Table 4. Similarly, no significant change in the amplitudes of platelet aggregation curves and in the TACs induced by collagen and adrenaline at the different time intervals could be detected (Table 4). Small changes between the various periods in the levels of TxB_2 formed by PRP, two min after collagen (10 μg/ml) stimulation, were noted during the study (Table 4).

PRP Sensitivity to Exogenous PGI_2

No difference in the sensitivity of PRP to exogenous PGI_2, evaluated as the IC_{50}s with respect to platelet aggregation induced by 4 μl/ml collagen, was found at the various time periods of the study. Levels of cAMP in PRP one min after incubation with exogenous PGI_2 also were not different at the various periods (Table 5).

DISCUSSION

A short term administration of PPC to healthy volunteers resulted in marked changes in the platelet membrane lipid composition. At the end of six weeks of PPC treatment, the total lipid and cholesterol (C) content of platelet

TABLE 4

Platelet Parameters

		Weeks		
		-4	0	+6 (end of treatment)	+10 (end wash out)
A. Whole blood aggregation	Amplitude[a]	11.3 ± 1.41	12.1 ± 0.91	12.7 ± 1.06	12.5 ± 0.61
B. PRP aggregation					
Collagen:	TAC[b]	0.46 ± 0.03	0.64 ± 0.09	0.71 ± 0.14	0.50 ± 0.09
	Amplitude[c]	14.1 ± 0.95	12.7 ± 0.83	15.1 ± 1.40	14.8 ± 0.86
Adrenaline:	TAC[b]	0.20 ± 0.06	0.27 ± 0.06	0.38 ± 0.10	0.25 ± 0.07
	Amplitude[c]	14.1 ± 1.11	11.9 ± 1.45	12.2 ± 0.98	14.2 ± 1.45
C. TxB_2 levels in collagen stimulated PRP[d]		556 ± 48	531 ± 73	571 ± 59	608 ± 48

Values are the average ± SEM.

[a]Amplitude (cm) of aggregation wave measured 8 min after stimulation with collagen (1 μg/ml).

[b]Threshold aggregation concentration (μg/ml).

[c]Amplitude (cm) of aggregation curve measured 5 min after stimulation with collagen (2 μg/ml) or adrenaline (1 μg/ml).

[d]Levels (pg/10^6 cells) of TxB_2 2 min after collagen (10 μg/ml) stimulation.

TABLE 5

Prostacyclin Activity on PRP

	Weeks		
	0	+6 (end of treatment)	+10 (end wash out)
cAMP (pmoles/10^6 platelets)[a]	36.7 ± 3.8	31.4 ± 4.3	29.9 ± 4.5
IC_{50} for PGI_2 (10^{-8}M)[b]	6.11 ± 0.99	8.42 ± 1.12	9.29 ± 0.79

Values are expressed as average ± SEM.

[a]cAMP levels measured 1 min after incubation with 0.8 10^{-8} M PGI_2.

[b]IC_{50} of PGI_2 for PRP aggregation induced by 4 μg/ml collagen.

membranes, expressed as the ratio to total proteins, were significantly reduced. The phospholipid (PL) content (as a percent of total lipids) was increased, and the C/PL ratio consequently reduced. PL of platelet membranes also were significantly richer in linoleic acid, compared to basal values. This suggests that administration of PC which is rich in linoleic acid results in the enrichment of platelet PL with this fatty acid.

The total lipid content of platelet membranes, in contrast, was markedly reduced following PPC, thus suggesting that incorporation of highly unsaturated PC into membranes may modify their lipid content. In this respect, it is of interest to note that platelet cholesterol, as related to platelet proteins, was reduced more than total lipids (a 25% versus a 17% reduction).

Platelet membrane modifications were not associated with corresponding major changes in plasma cholesterol levels. This last finding possibly is in contrast with previous experimental data, indicative of a direct correlation between platelet cholesterol content and plasma cholesterol levels (17). These findings were, however, based on the well known inability of platelets to synthesize cholesterol (18). On the other hand, the modulation of cholesterol exchanges between plasma and platelets may be regulated by poorly documented receptor-mediated mechanisms (19) and, moreover, these previous studies, particularly based on platelet cholesterol enrichment via incubation with liposomes, failed to investigate thoroughly the platelet lipid composition (20). A potential contributor to these exchanges may be found in HDL (21), and the increased plasma HDL-C levels after PPC treatment may be involved in the induction of the observed platelet membrane compositional changes.

Outside of the HDL-C increase, other plasma lipid-lipoprotein changes after PPC treatment were unremarkable.

During the wash out period only a significant fall of total and LDL cholesterol levels, as well

as of LDL/HDL ratio, was detected. Although this finding cannot be explained directly, the possibility that plasma cholesterol is either actively taken up by tissues or differently metabolized during wash out may be considered. Similarly, the marked effects of PPC on plasma glucose had not been described previously. However, in view of the highly viscous preparation given to volunteers, it cannot be excluded that the glucose reduction may be ascribed to a fiber-like activity of oral PPC (22).

The biochemical modifications of platelet membranes were not associated with any change of platelet function parameters, such as aggregatory responses to various stimuli, TxB_2 formation and sensitivity to PGI_2. Experimental and clinical evidence for an increased platelet sensitivity to aggregating agents and TxB_2 formation of cholesterol enriched platelets has been provided (20). On the other hand, as previously indicated, the available information does not take into account changes in the cholesterol/total lipid ratio and particularly the increased PL content, the latter being the major change following PPC treatment, coming from our study. It is possible that platelet function parameters may be modified by PPC in high risk patients, where an altered pattern of aggregation and TxB_2 formation is present (23).

In conclusion, our study in normal volunteers shows that oral PPC administration significantly affects platelet membrane lipid composition. A particularly significant reduction in the proportion of cholesterol in total lipids was recorded. Only minor plasma lipoprotein modifications and essentially no change in the platelet function parameters were recorded. Whether the membrane changes following PPC are specific for platelets or represent a generalized effect in different cell systems requires further investigation.

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Lipids and Lipid Antioxidant Systems in Developing Eggs of Salmon (*Salmo salar*)

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ABSTRACT

Lipid class and fatty acid analyses were carried out on developing salmon eggs at four clearly defined pre-feeding stages, namely, fertilization, eyed egg stage (50 days), hatching (yolk sac fry, 98 days) and swim up fry (138 days).

Measurements of components of the system considered to be involved in defense of cells against lipid peroxidation (glutathione peroxidase, EC 1.11.1.9, glutathione S-transferase, EC 2.5.1.18, reduced glutathione [GSH], α-tocopherol and ascorbic acid) were made at the same time. Levels of triacylglycerol decreased markedly during development, but there were few changes in fatty acid composition, indicating a non-selective utilization of fatty acids. Phosphatidylcholine was the dominant polar lipid (> 94% by weight) in fertilized eggs. It was used preferentially during development so that in swim up fry the ratio phosphatidylcholine:phosphatidylethanolamine approached that found in fish muscle. Amounts of docosahexaenoic acid and arachidonic acid in polar lipids were significantly greater ($p < 0.01$) in swim up fry than in fertilized eggs.

Activities of the two enzymes were very low in the fertilized egg and remained low until hatching, when there was a concerted increase in their activity and in the concentration of GSH. Egg tocopherol concentrations decreased significantly during development, but whole body concentrations in swim up fry were not dissimilar from those in normal juvenile fish. Ascorbic acid, on the other hand, declined to very low levels in swim up fry; the restoration of this vitamin during first feeding seems vital to the well being of the fish.

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INTRODUCTION

Fish lipids are characterized generally by the presence of large quantities of highly unsaturated fatty acids. This is true of the lipids translocated to and deposited in maturing eggs (1). These eggs frequently develop at low temperatures (from 4-5 C upward), where maintenance of membrane fluidity may pose constraints on the fatty acids incorporated into biomembranes (2). Consequently any changes in proportions of lipid classes and of fatty acids occurring in fish eggs during development are of great interest. The prolonged period of embryonic development in certain fish, such as Atlantic salmon (*Salmo salar*), lends itself to the study of changes in cellular components (and in the activities of enzymes) at more clearly defined stages than is the case with more rapidly developing organisms.

Unsaturated fatty acids are susceptible to attack by free radical metabolites of oxygen, leading to oxidative chain reactions and cell damage. The dangers are exemplified by outbreaks of disease related to fatty acid autoxidation in young salmonids (3); these dangers may increase at lower temperatures (5-6 C) (4). During the development of salmonid eggs, aerobic metabolism has been shown to increase continuously (5) so that potentially toxic derivatives of oxygen will arise and must be controlled.

While the cell defense system against oxygen toxicity includes a number of enzymes such as superoxide dismutase and catalase, glutathione peroxidase (EC 1.11.1.9) and glutathione S-transferase (EC 2.5.1.18, possessing so-called selenium-independent glutathione peroxidase activity) have been assigned special roles in combatting peroxidative attack on unsaturated fatty acids. Other levels in this system include α-tocopherol, a terminator of free radical chain reactions, and possibly ascorbic acid which recently has been shown to reduce in vivo lipid peroxidation in guinea pigs injected with carbon tetrachloride (6).

The object of the present work was to follow changes in lipid class and fatty acid composition at clearly defined stages of developing eggs of the Atlantic salmon along with temporal changes in certain components of the system concerned with prevention of oxidative damage to fatty acids, glutathione peroxidase activity, glutathione S-transferase activity and concentrations of reduced glutathione (GSH), α-tocopherol and ascorbic acid.

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MATERIALS AND METHODS

Salmon Eggs

These were obtained from Landcatch, Lochgilphead, Argyll. Eggs were stripped and fertilized by conventional procedures and immediately transported to the laboratory aquarium in Aberdeen. They were placed in hatching equipment (trough, boxes, screen) obtained from Ewos Baker, Bathgate, Scotland, and maintained in running tap water supplied via a header tank. Over the 140-day period from fertilization to swim up fry, water temperature varied between 4 and 9 C. Analyses were performed at clearly recognizable development stages, namely at fertilization, eyed stage (50 days), hatching (yolk sac fry, 98 days) and swim up stage (138 days).

Enzyme, Vitamin and Thiol Assays

Eggs or embryos (10-35 depending on measurement being made and stage of development) were removed from the hatching box with gentle suction through a glass tube. They were placed on absorbent tissue to remove water, then immediately weighed and extracted. Glutathione peroxidase activity, glutathione S-transferase activity and tocopherol concentration were measured as described previously (7).

Ascorbic acid was measured by an ascorbic acid oxidase method specific for L-ascorbic acid using a kit supplied by The Boehringer Corporation, United Kingdom; appropriate recovery tests and internal standards were applied. Extraction, resolution and measurement of those thiols present was by the method of Newton et al. (8).

Lipid Class and Fatty Acid Analysis

Total lipid was extracted by the method of Folch et al. (9) and measured gravimetrically. Between analyses the chloroform extract was purged with argon and stored at -20 C. Lipid class analysis was carried out on an Iatroscan TH-10 (Iatron Laboratories, Tokyo, Japan) operated in conjunction with a Hewlett-Packard 3390A recording integrator. Neutral lipids were separated in hexane/diethyl ether/formic acid (85:15:0.04, v/v/v) and polar lipids in chloroform/methanol/water (70:35:3.5, v/v/v). Quantitation of lipid classes was effected through the use of calibration curves prepared from a composite standard having a composition similar to the samples under analysis as described by Fraser et al. (10).

Polar and neutral lipids were separated by thin layer chromatography (TLC) on silica gel 60 plates using hexane/diethyl ether/acetic acid (80:20:2, v/v/v) as solvent. The separated lipids were eluted with chloroform/methanol (2:1, v/v) and methyl esters of fatty acids prepared by acid catalyzed transmethylation (11), then purified by TLC. Fatty acid methyl esters were resolved and measured in a Carlo Erba HRGC 41 series gas chromatograph equipped with a FFAP capillary column (50 m × 0.04 mm i.d.) and using hydrogen as carrier gas. Identification was by comparison with known standards and by reference to published data (12). All analyses were carried out on three batches of eggs or fry at each stage.

Statistical Analyses

Data were subjected to analysis of variance and a multiple range test (13).

RESULTS

Values are expressed as amount or activity of component per ontogenetic unit, this being one egg or the development stage originating from one egg. There is a significant decrease in weight ($p < 0.05$) of each ontogenetic unit over the 140-day period to swim up fry (Fig. 1). However, the loss in dry matter during the final phase is partly masked because over this time period a significant increase in water content occurs (Fig. 1).

Activities of GSH S-transferase and of GSH peroxidase are extremely low in the fertilized egg (Fig. 2) and remain low until hatching. The large increase in activities manifest then continues to the fry stage. These values are approxi-

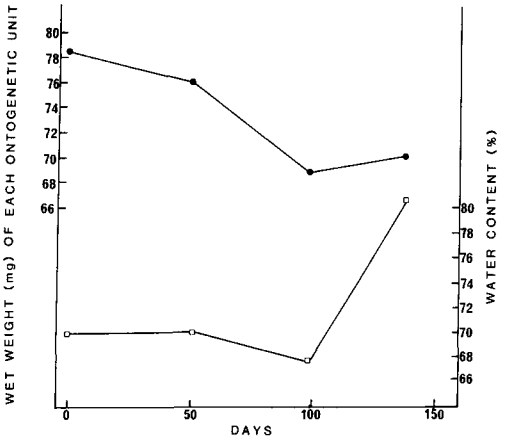

FIG. 1. Changes in wet weight (●——●) and in water content (□——□) of salmon eggs during development. Measurements were made at fertilization, eyed egg stage (50 days), hatching (yolk sac fry, 98 days) and at swim up fry (138 days). Values for wet weight are per ontogenetic unit (one egg or the development stage arising from one egg).

mately 5- to 10-fold lower than those found in the liver of growing (10-100 g) trout. Activities in some embryonic organs probably are much higher than those found in the whole ontogenetic unit.

The only thiols detected were reduced glutathione and cysteine. There was a rapid increase in GSH concentration at hatching (Fig. 2) and although the concentrations decreased at the swim up stage in absolute (ontogenetic unit) terms, the concentration in dry matter terms was significantly higher at this time.

Levels of lipid declined steadily throughout the development period studied (Table 1). Ascorbic acid concentration declined from the eyed egg stage to very low levels in the swim up fry. Significant changes in tocopherol level occurred only during the final period (Table 1) at a time when antioxidant enzyme activities and co-substrate (GSH) concentration were increasing.

The main change in neutral lipids during egg development was the marked fall in triacylglycerols, the main energy substrate over this period (Table 2). The presence of partial acylglycerols in yolk sac and swim up fry may indicate a more rapid rate of triacylglycerol breakdown at this time. Free fatty acids began to appear in the later stages of development, indicating that lipolysis was tending to exceed fatty acid catabolism although the absence of any detectable free fatty acids in yolk sac fry indicates that the two processes are closely balanced.

Phosphatidylcholine (PC) was the dominant polar lipid comprising over 90% of the total in the fertilized egg; phosphatidylethanolamine (PE, 5% of the total) was the only other phosphatide present in appreciable amount. The large decline in PC level as development proceeded was indicative of its use as an energy source and, paralleling changes in triacylglycerols, lysophosphatidylcholine was present in yolk sac and swim up fry (Table 2). As the concentration of other phosphatides altered little during development, a marked change in relative amounts occurred, the PC:PE ratio falling from 17 in the fertilized egg to less than six in swim up fry.

Although a reduction of more than 50% occurred in the level of triacylglycerols during development, few relative changes in fatty acid composition occurred in this component (Table 3). The absence of such changes is indicative of mainly non-selective catabolism of fatty acids. The principal (n-6) fatty acid was linoleic acid (18:2 [n-6]), with little arachidonic acid (20:4, [n-6]) present.

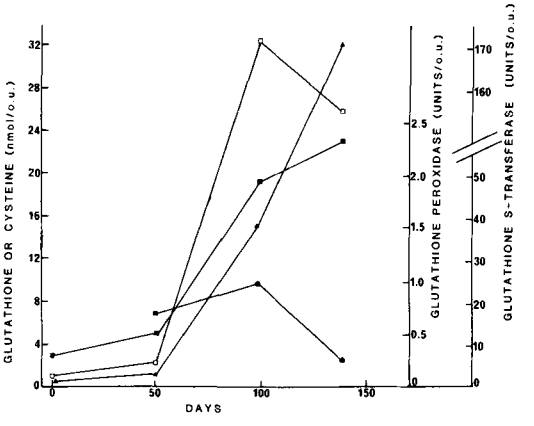

FIG. 2. Changes in cysteine (●——●) and reduced glutathione (□——□) content and in the activities of glutathione peroxidase (■——■) and of glutathione transferase (▲——▲) in salmon eggs during development. Measurements were made at fertilization, eyed egg stage (50 days), hatching (yolk sac fry, 98 days) and at swim up fry (138 days). Enzyme units are μmole substrate converted or thioester bond formed/hour/ontogenetic unit.

TABLE 1

Lipid, Tocopherol and Ascorbic Acid Levels in Developing Eggs of Atlantic Salmon

	Fertilized eggs	Eyed eggs	Yolk sac fry	Swim up fry
Days since fertilization	0.5	50	98	138
Lipid (mg)	7.02 ± 0.07[a]	5.90 ± 0.02[b]	5.23 ± 0.08[c]	3.50 ± 0.03[c]
α-Tocopherol (μg)	3.10 ± 0.13[a]	3.15 ± 0.06[a]	2.93 ± 0.05[b]	2.29 ± 0.11[c]
Ascorbic acid (μg)	2.90 ± 0.10[a]	3.17 ± 0.17[b]	2.16 ± 0.05[c]	0.71 ± 0.04[d]

Values given are per ontogenetic unit (one egg or the development stage arising from one egg). They are mean values of measurements on 3 samples at each stage together with their standard error.

Values in the same row with different superscript letters are significantly different ($p < 0.05$).

TABLE 2

Lipid Class Composition of Developing Atlantic Salmon Eggs

	Fertilized eggs	Eyed eggs	Yolk sac fry	Swim up fry
Days since fertilization	0.5	50	98	138
Sterol esters	0.21 ± 0.01	0.22 ± 0.01	0.28 ± 0.04	0.20 ± 0.02
Triacylglycerol	3.37 ± 0.04[a]	2.66 ± 0.01[b]	2.35 ± 0.03[c]	1.44 ± 0.10[d]
Cholesterol	0.33 ± 0.01	0.27 ± 0.02	0.27 ± 0.02	0.24 ± 0.03
Free fatty acids	ND	0.065 ± 0.024	ND	0.191 ± 0.081
Partial acylglycerol	ND	ND	0.027 ± 0.004	0.025 ± 0.005
Phosphatidylethanolamine	0.17 ± 0.01[a]	0.13 ± 0.01[b]	0.22 ± 0.003[c]	0.19 ± 0.02[a]
Phosphatidylserine + phosphatidylinositol	ND	0.040 ± 0.001[a]	0.060 ± 0.003[b]	0.048 ± 0.002[c]
Phosphatidylcholine	2.89 ± 0.06[a]	2.51 ± 0.03[b]	1.97 ± 0.04[c]	1.08 ± 0.07[c]
Sphingomyelin	0.018 ± 0.002[a]	0.017 ± 0.002[a]	0.052 ± 0.001[b]	0.049 ± 0.0027
Lysophosphatidylcholine	ND	ND	0.026 ± 0.009	0.053 ± 0.013

Values given are mg per ontogenetic unit at each stage. Mean values of measurements on 3 samples at each stage together with their standard error.

Values in the same row with different superscript letters are significantly different ($p < 0.05$). Where no superscript letter appears there are no significant differences.

ND, not detected.

TABLE 3

Fatty Acids of Biochemical Interest in Triacylglycerols of Salmon Eggs During Development

	Fertilized eggs	Eyed eggs	Yolk sac fry	Swim up fry
14:0	2.95 ± 0.06	2.99 ± 0.02	3.01 ± 0.20	2.87 ± 0.05
16:0	11.43 ± 0.20	10.82 ± 0.11	11.46 ± 0.59	10.49 ± 0.33
16:1 (n-7)	9.89 ± 0.28	10.10 ± 0.24	10.02 ± 0.70	9.14 ± 0.59
18:0	3.12 ± 0.07	3.02 ± 0.05	3.23 ± 0.05	3.65 ± 0.13
18:1 (n-9)	30.86 ± 0.59	30.16 ± 0.16	30.17 ± 0.34	27.19 ± 1.01
18:1 (n-7)	4.95 ± 0.05	4.53 ± 0.31	4.63 ± 0.42	5.86 ± 0.61
18:2 (n-6)	4.53 ± 0.13	4.38 ± 0.09	4.48 ± 0.03	4.25 ± 0.11
18:3 (n-3)	1.02 ± 0.04	0.99 ± 0.01	1.00 ± 0.01	0.98 ± 0.04
20:1 (n-9)	2.60 ± 0.13	2.74 ± 0.09	2.63 ± 0.02	2.81 ± 0.12
20:4 (n-6)	0.70 ± 0.09	0.71 ± 0.02	0.71 ± 0.01	0.90 ± 0.01
20:4 (n-3)	2.21 ± 0.02	2.16 ± 0.01	2.06 ± 0.04	2.15 ± 0.02
20:5 (n-3)	6.03 ± 0.07	6.05 ± 0.06	5.85 ± 0.23	6.21 ± 0.13
22:5 (n-3)	3.46 ± 0.01	3.66 ± 0.03	3.20 ± 0.14	3.98 ± 0.06
22:6 (n-3)	11.43 ± 0.14	12.24 ± 0.09	10.92 ± 0.43	14.72 ± 1.31
Total (n-6) polyunsaturates	5.23	5.09	5.19	5.15
Total (n-3) polyunsaturates	24.15	25.10	23.03	28.04
(n-3)/(n-6)	4.62	4.93	4.44	5.45

Values are per cent by weight. Mean values on 3 samples at each stage together with their standard error.

Much higher levels of (n-3) fatty acids were present in polar than in triacylglycerols (Table 4). The total concentration of these fatty acids changed little during development, but amounts of docosahexaenoic acid increased significantly ($p < 0.01$) in the swim up fry compared with fertilized eggs. The main (n-6) fatty acid was 20:4 (n-6), and this also increased significantly ($p < 0.01$) during development.

DISCUSSION

The fish egg contains levels of nutrient that must meet demands for both energy and growth of the embryo and of the resultant fry up to the stage of yolk sac absorption and first feeding. Triacylglycerols commonly serve as a major energy reserve, and these were heavily used during development in the present experiment.

TABLE 4

Fatty Acids of Biochemical Interest in Polar Lipids of Salmon Eggs During Development

	Fertilized eggs	Eyed eggs	Yolk sac fry	Swim up fry
14:0	1.07 ± 0.04	0.54 ± 0.05	0.78 ± 0.16	0.78 ± 0.09
16:0	14.49 ± 0.62	13.34 ± 0.13	14.91 ± 0.21	15.29 ± 0.41
16:1 (n-7)	2.44 ± 0.12	1.48 ± 0.07	2.37 ± 0.29	1.93 ± 0.05
18:1 (n-9)	12.79 ± 0.32	11.00 ± 0.09	11.71 ± 0.49	11.16 ± 0.27
18:1 (n-7)	4.25 ± 0.08	4.43 ± 0.01	4.15 ± 0.05	3.57 ± 0.04
18:2 (n-6)	1.43 ± 0.09	1.23 ± 0.07	1.37 ± 0.08	1.36 ± 0.06
18:3 (n-3)	0.42 ± 0.08	0.36 ± 0.05	0.18 ± 0.03	0.17 ± 0.04
20:1 (n-9)	3.25 ± 0.15	3.54 ± 0.16	3.03 ± 0.15	1.91 ± 0.07
20:4 (n-6)	2.18 ± 0.08	2.33 ± 0.02	2.40 ± 0.04	2.79 ± 0.06
20:4 (n-3)	1.30 ± 0.05	1.13 ± 0.08	0.93 ± 0.03	0.82 ± 0.05
20:5 (n-3)	9.70 ± 0.03	10.30 ± 0.12	9.93 ± 0.14	8.53 ± 0.07
22:5 (n-3)	5.53 ± 0.08	6.24 ± 0.06	5.40 ± 0.15	4.56 ± 0.41
22:6 (n-3)	25.50 ± 0.53	29.50 ± 0.23	28.73 ± 0.84	31.60 ± 1.01
Total (n-6) polyunsaturates	3.61	3.56	3.77	4.15
Total (n-3) polyunsaturates	42.25	47.53	45.17	45.68
(n-3)/(n-6)	11.76	13.35	11.98	11.00

Values are per cent by weight. Mean values on 3 samples at each stage together with their standard error.

The presence of free fatty acids in eyed eggs and swim up fry was interpreted as indicating that the rate of release of fatty acids from triacylglycerols (and PC) may exceed rates at which they are catabolized. Another possibility is that they are post mortem artifacts arising from autolysis. Such artifacts are not easy to avoid, and any increase in level of a trace constituent such as free fatty acids frequently is taken to indicate autolysis (14). In this context it may be noted that the eggs and fry were living at temperatures below 10 C, and no dissection was required prior to homogenization; the organisms were simply blotted and weighed rapidly.

Large amounts of PC also were used as an energy source, a very large excess of PC over PE being present in fertilized eggs. The ratio PC:PE of 17 considerably exceeded the value of 9.6 reported for trout roe (15). The preferential utilization of PC during development reduced this value to approximately five in swim up fry, a value more nearly approaching that reported for roe of several marine fish (1) and for the muscle of marine fish (16).

Apart from 22:6 (n-3), the only component fatty acid of the polar lipids which increased significantly in relative amount over the period was 20:4 (n-6). It is also of interest that, while the proportion of (n-6) fatty acids in triacylglycerols (5% at all stages) exceeds that in polar lipids (3-4%), the bulk of (n-6) in triacylglycerols is 18:2 (n-6), while in polar lipids most (n-6) is present as 20:4 (n-6). These observations are not inconsistent with the specific role proposed for 20:4 (n-6) in biomembranes, especially of salt secreting tissues (17). Although linolenic acid (18:3 [n-3]) is the principal essential fatty acid (EFA) for salmonids, and Yu et al. (18) have grown rainbow trout through a generation on a semi-purified diet containing 1% linolenic acid as the sole EFA, the eggs produced did contain some 20:4 (n-6) that was attributed to trace amounts (probably of 18:2 [n-6]) in dietary components.

Concentrations of long chain highly unsaturated fatty acids (> 20:5) are very high in the polar lipids of salmon eggs, and these generally are seen as essential to maintenance of membrane fluidity. Besides this general requirement it is noteworthy that nervous tissue of mammals and retina in particular is characterized by unusually large proportions of 22:6 (n-3) (19). The nervous system in salmonids may equally impose additional demands for this fatty acid as it develops.

Of the two GSH-dependent enzymes measured, we were not able previously to detect GSH preoxidase activity in partially purified GSH S-transferase from rainbow trout. However, this enzyme did inhibit Fe^{3+}-induced microsomal lipid oxidation in vitro (7) and so may contribute to the fatty acid antioxidant system. The two enzymes show a concerted increase in activity during the later stages of development. The (whole body) activity attained by either enzyme is appreciably less (5- to 10-fold) than that present in the livers of normal

trout (20-100 g or more), and the likelihood is that in embryonic organs activity does not attain levels present in larger trout until some time after feeding has begun. Temporal increases in the concentration of co-substrate GSH also closely parallel increases in enzyme activity so that an efficient system for removal of peroxidase is established soon after hatching.

Tocopherol levels decreased significantly during the final period of development. Nevertheless, whole body levels were, in swim up fry, comparable with concentration in livers of normal trout (about 30 μg/g wet weight) given dietary tocopherol.

Ascorbic acid was measured in the context of lipid oxidation for reasons stated earlier. Amounts present in swim up fry were very low following a rapid decrease during the later stages of development. The restoration of ascorbic acid levels upon first feeding may be critical for the well being of the fish.

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Gamma Linolenic Acid Attenuates Cardiovascular Responses to Stress in Borderline Hypertensive Rats

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ABSTRACT

The purpose of the present study was to investigate the effects of gamma linolenic acid (GLA) on cardiovascular responses to psychosocial stress (isolation) and to pressor hormones in the genetically borderline hypertensive rat (SHR × WKY). Adult male SHR × WKY were divided into two groups following five weeks of group housing. One group (GLA) received eight weeks constant flow osmotic pumps releasing 0.04 mg GLA in olive oil/kg-hr, while the second group received dummy pumps (DUM). One week following pump implantation, each group was divided into two subgroups and exposed to a four-week experimental period of either continued group housing (no stress) or isolation (stress). A two-week recovery period of group housing followed the experimental period. Blood pressure and heart rate were determined weekly by the tail cuff technique. At the end of the recovery period, animals in the no stress condition were anesthetized and received an arterial cannula for NOR and ANG infusion and direct BP recording. Then the responses to an ED_{50} of NOR and ANG were determined. All animals were then killed for determination of heart weight and adrenal weight. All groups had mean control period systolic BP values ranging from 143-146 mm Hg. In the no stress condition, neither GLA nor DUM altered BP over the course of the study. However, BP increased in the DUM group during all four weeks of the isolation period vs the control period ($p<0.01$), whereas BP increased only in week 1 in the GLA group ($p<0.05$). Heart rate increased during stress in the DUM group ($p<0.05$), but not in the GLA group. Vascular reactivity to NOR was unaffected by GLA administration. In contrast, GLA increased the duration of the pressor response to ANG ($p<0.01$), but tended to decrease the magnitude of the pressor response ($p<0.09$) vs DUM. GLA had no effect on heart weight/body weight ratio. Adrenal weight/body weight ratio was lower in the DUM/no stress group than all other treatment groups.

These data indicate that GLA administration attenuates the cardiovascular responses to chronic stress in animals with a genetic predisposition to hypertension, in the absence of an effect on resting BP. They also demonstrate a limitation of GLA effect, in mature animals, to epigenetic pressor factors. Furthermore, GLA action may involve an alteration of the cardiovascular responses to ANG, but not NOR. These findings suggest that GLA may be useful in preventing the neurogenic triggering of hypertension by chronic stress in genetically stress-sensitive animals.

Lipids 20:573-577, 1985.

INTRODUCTION

The administration of gamma linolenic acid (GLA), a metabolite of linoleic acid, has been demonstrated to be effective in attenuating elevations of blood pressure (BP) in several animal models. Mills and Ward recently reported that purified GLA prevented the development of stress-induced borderline hypertension in genetically normotensive rats, in the absence of an effect on resting, pre-stress BP (1). In addition, Hoffman and Förster observed that GLA, in the form of evening primrose oil, attenuated the development of hypertension in the genetically hypertensive SHR (2). A fundamental difference between these two models is that the hypertension produced by stress in the genetically normotensive rats reverses itself following cessation of the stress and does not lead to a chronic, irreversible hypertensive condition. In contrast, hypertension in the SHR is chronic and irreversible. These two animal models represent extremes in the continuum of environmental/hereditary factors involved in the regulation of BP.

An animal model in which environmental factors, such as stress, interact with hereditary factors in the pathogenesis of hypertension would be a useful tool to elucidate the mechanisms and limitations of GLA activity. Lawler et al. have reported the development of such an animal (3,4), which is produced by backcrossing the SHR with its normotensive Wistar Kyoto (WKY) parent strain. The F_1 generation from this mating develops a chronic borderline hypertension (140-155 mm Hg) which does not increase unless the animal is chronically stressed. Stress then acts as a neurogenic trigger producing a chronic, irreversible hypertension similar to that of the SHR.

The purpose of the present study was to examine the effectiveness of GLA in attenuating the cardiovascular responses to psychosocial stress in the genetically borderline hypertensive rat, in an attempt to determine the effects of

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GLA on genetic and epigenetic hypertensive influences. In this study, BP, heart rate (HR), heart weight and vascular reactivity to exogenous norepinephrine (NOR) and angiotensin (ANG) were examined.

MATERIALS AND METHODS

Animals

The study utilized 33 F_1 generation male offspring of female SHR and male WKY rats. Both inbred parent strains were purchased from Taconic Farms (Germantown, New York). All F_1 generation were born within seven days of each other and weaned at 21 days of age. Post-weaning, all rats were group housed four per cage for a five-week acclimation period prior to the start of the study. During the course of the study all animals were maintained in a temperature (21 ± 1 C) and light cycle controlled (14L: 10D) room and were given rat chow and tap water ad lib.

Blood Pressure Determination

During the six-week study, systolic BP was measured indirectly using the tail cuff technique (1,5). Animals were pre-warmed for eight min in a 35 C incubator and then placed in a conical cloth holder during the measurement. Each recorded BP value represents the mean of 10 determinations taken over a 2-min period after stabilization of the tracing. Prior to the study all animals had three BP determinations made in order to accustom them to the procedure and to obtain a baseline.

During the determination of cardiovascular reactivity to NOR and ANG, BP was monitored directly via an arterial cannula inserted to the level of the renal artery.

Agents Administered

GLA was administered by means of eight-week, constant flow (1.15 μl/hr), osmotic mini-pumps (modified Alzet 2ML4) implanted under halothane anesthesia at the start of the study. Gas chromatographic analysis of the GLA (Sigma) demonstrated purity greater than 99%. The olive oil carrier for the GLA was shown by gas chromatography to contain 0.4% α-linolenic acid and 9.6% linoleic acid, and has been shown to have no effect on BP at the rate of administration used in the present study (1). Furthermore, pilot studies conducted in our laboratory suggest that neither olive oil, released by osmotic pumps, nor dummy pumps have any effect on BP (unpublished observation).

Protocol

Following the five-week acclimation period, animals were divided into four treatment groups. At this time, two of the groups received osmotic pumps releasing 0.04 mg GLA in olive oil/hr, while two others received dummy pumps (DUM). All animals then remained in group housing for an additional one-week control period in which baseline BP and HR were recorded. One week post surgery, one group of GLA-treated rats (n=8) and one DUM group (n=8) were placed in isolation cages (stress) for a four-week experimental period. In addition, one GLA group (n=8) and one DUM group (n=9) remained in groups (no-stress) for a four-week period as age-matched controls.

A two-week recovery period followed the experimental period. During this time all isolated animals were returned to their original groups. Blood pressure, HR and body weights were determined on days 1, 7, 14, 21 and 28 after the start of the experimental period. In addition, these parameters were measured 1, 7 and 14 days into the recovery period.

One day following the final BP determination, unstressed animals were anesthetized with halothane, and their cardiovascular reactivity to an i.a. ED_{50} of NOR (4.3 μg) and ANG (1.25 μg) was assessed. All animals were killed immediately following the recovery period and their hearts and adrenals dissected out for weighing.

Statistics

Blood pressure, HR and body weight data were analyzed using a two-way ANOVA. Where a difference of $p<0.05$ was achieved, specific points of difference were determined using a planned comparisons technique (6). Data on organ weight was analyzed using a one-way ANOVA. Vascular reactivity was analyzed using the Student t-test.

Results BP, HR and Body Weight Responses to Isolation

Systolic BP responses to the experimental procedures are shown in Figure 1. Mean control period BP values of the four treatment groups ranged from 143-146 mm Hg. In the no-stress condition there were no significant changes in BP in either group, and GLA had no effect on BP in comparison to the DUM group.

In contrast, social isolation significantly increased BP in the DUM group in each of the four weeks of the stress period ($p<0.01$ vs pre-isolation). During the recovery period, BP in the DUM group returned to levels which were not statistically different from pre-isolation values (Fig. 1b).

Animals receiving GLA demonstrated a transient increase in BP after one week of isolation ($p<0.05$ vs pre-isolation), which returned to pre-isolation levels by week 2 and remained

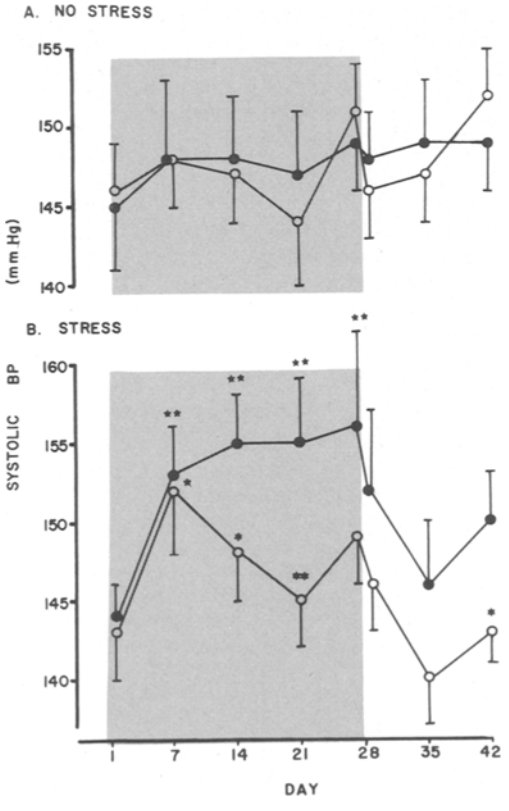

FIG. 1. Systolic blood pressure of group housed male SHR × WKY rats before, during and following a 4-week experimental period of (A) group housing (no stress), or (B) isolation (stress). Animals were administered either dummy pumps (●—● DUM) or 0.04 mg GLA in olive oil/hr (○—○ GLA) by osmotic minipump implanted i.p. 2 weeks prior to the experimental period. Values represent $\bar{x}$ ± S.E. Shaded area represents experimental period. * $p<0.05$ vs DUM; ** $p<0.01$ vs DUM; ★ $p<0.05$ vs Day 1; ★★ $p<0.01$ vs Day 1.

there for the duration of the study. Furthermore, BP of the GLA group was significantly lower than that of the DUM group after two ($p<0.05$) and three ($p<0.01$) weeks of isolation and after two weeks of recovery ($p<0.05$).

Heart rate responses to the experimental procedures may be found in Table 1. There were no significant changes in heart rate in either the unstressed groups or the stressed GLA group. In contrast, stress significantly increased heart rate from pre-stress values in DUM animals on day 21 of isolation ($p<0.05$). In addition, on day 28 of stress, GLA administration was associated with a significantly lower heart rate than in stressed DUM animals ($p<0.05$).

Body weights and rate of weight gain were unaffected by the experimental procedures and were not statistically different among the four treatment groups (data not shown). All groups tended to gain weight steadily during the course of the study, except for a tendency to decline transiently after one day of regrouping at the start of the recovery period. Following this event, all groups returned to their original growth curves.

Organ Weights and Vascular Reactivity

The effects of stress and fatty acid administration on paired adrenal and heart weights (wet tissue weight) are shown in Table 2. Both stressed groups, as well as unstressed, GLA-supplemented animals demonstrated significantly greater adrenal weight/body weight ratios than the unstressed DUM groups. In contrast, treatment had no effect on heart weight.

Cardiovascular responses to NOR and ANG in unstressed DUM and GLA groups are illustrated in Table 3. GLA administration had no effect on the responses to NOR, but significantly increased the duration of response to

TABLE 1

Mean Heart Rates (bpm ± SE) of Group Housed Male SHR × WKY Rats Before, During and After 4-Week Period of Either Group Housing (no stress) or Isolation (stress)[a]

	Control	Experimental				Recovery		
	Day 1	Day 7	Day 14	Day 21	Day 29	Day 30	Day 35	Day 42
DUM/No stress (n=8)	380 ± 17	383 ± 7	391 ± 14	418 ± 17	411 ± 6	365 ± 20	395 ± 9	354 ± 11
DUM/Stress (n=8)	394 ± 18	391 ± 10	404 ± 19	443 ± 15[b]	428 ± 14	401 ± 12	376 ± 9	380 ± 14
GLA/No stress (n=8)	377 ± 11	393 ± 8	393 ± 13	391 ± 15	399 ± 9	363 ± 13	373 ± 11	354 ± 14
GLA/Stress (n=9)	398 ± 18	399 ± 18	404 ± 19	412 ± 13	384 ± 7[c]	400 ± 13	393 ± 13	388 ± 10

[a]Animals received either dummy pumps (DUM) or 0.04 mg GLA in olive oil/hr (GLA) by i.p. osmotic minipump throughout the study.

[b]$p<0.05$ vs Day 1.

[c]$p<0.05$ vs DUM/Stress.

ANG ($p<0.01$) vs the DUM group. Furthermore, GLA tended to decrease the systolic ($p<0.08$) and diastolic ($p<0.09$) BP response to ANG in comparison to DUM animals.

DISCUSSION

It has been suggested that stress is an etiological factor in the pathogenesis of essential hypertension in certain individuals (7,8). Furthermore, it has been proposed that those individuals in which stress may be a precipitating factor may have a genetic sensitivity or susceptibility to the maladaptive effects of stress (9). The animal model used in the present study approximates this mechanism of pathogenesis in that chronic stress acts as a neurogenic trigger of hypertension in a genetically sensitive, or predisposed, borderline hypertensive animal.

Chronic isolation of group raised animals, in the present study, produced a chronic elevation of BP in control (DUM) animals, which was reversed after one week by concomitant GLA infusion. In contrast to several previous reports in the literature, stress did not act as a neurogenic trigger for an irreversible hypertensive condition in the present study. The apparent discrepancy between the current study and previous reports may be due to variations in the duration (4 weeks vs 12 weeks) and intensity (isolation vs conflict and shock) of stress between the studies. The nature of the stressor used in the current study was apparently a subthreshold stimulus for the neurogenic triggering effect.

Another observation of the present study was that GLA administration attenuated the pressor response to an environmental stimulus without affecting genetic influences on resting BP. Although the mechanism of action of GLA in this instance is not clear, there are several possibilities. Administration of GLA in both animals (10,11) and man (12) is associated with an increase in dihomo-GLA stores as well as an increase in ability to synthesize E-series prostaglandins (PGE). It is possible that the BP elevation normally seen in response to stress results, in part, from the suppression of $\Delta 6$ desaturase activity by the stress hormones epinephrine and corticosterone, and the resultant decrease in conversion of linoleic acid to dihomo-GLA and arachidonic acid (13,14). Supplementation with GLA during stress may circumvent this metabolic blockade, enabling the animal to increase its PGE production. An enhanced PGE production during stress could then prevent an increase in BP via its vasodepressor actions (15,16).

As in earlier reports (1), the current study suggests that the effect of GLA on BP in this model is seen only during the stress, when sympathetic activity is heightened. This suggests that GLA is acting by decreasing either the

TABLE 2

Heart and Paired Adrenal Gland Weights of SHR X WKY Rats Receiving Either Dummy Pumps (DUM) or Pumps Releasing 0.04 mg GLA in Olive Oil/hr (GLA), Measured 2 Weeks Following the Cessation of a 4-Week Period of Either Group Housing (no stress) or Isolation (stress) Conditions

	Adrenal weight (g/100 g BW)	Heart weight (g/100 g BW)
DUM/no stress (n=8)	0.009 ± 0.001	0.269 ± 0.006
DUM/stress (n=8)	0.013 ± 0.001[a]	0.284 ± 0.006
GLA/no stress (n=4)	0.012 ± 0.001[b]	0.271 ± 0.016
GLA/stress (n=8)	0.012 ± 0.001[b]	0.278 ± 0.009

($\bar{x}$ ± SE).

[a]$p<0.01$ vs DUM/no stress.

[b]$p<0.05$ vs Dum/no stress.

TABLE 3

Effects of Dummy Pumps (DUM) and GLA, Administered as Previously Described, on the Cardiovascular Reactivity of Male Borderline Hypertensive Rats (SHR X WKY) to an i.a. ED_{50} of NOR and ANG Following 6 Weeks of Group Housing (no stress)

	DUM (n=8)	GLA (n=9)	p
Duration of response to NOR (sec)	255 ± 35	288 ± 24	NS
Δ systolic BP following NOR (mm Hg)	41 ± 4	47 ± 7	NS
Δ diastolic BP following NOR (mm Hg)	46 ± 8	42 ± 4	NS
Δ heart rate following NOR (min^{-1})	68 ± 19	61 ± 23	NS
Duration of response to ANG (sec)	162 ± 12	223 ± 15	<0.01
Δ systolic BP following ANG (mm Hg)	41 ± 4	31 ± 3	<0.08
Δ diastolic BP following ANG (mm Hg)	41 ± 4	31 ± 4	<0.09
Δ heart rate following ANG (min^{-1})	38 ± 24	47 ± 16	NS

($\bar{x}$ ± SEM).

sympathetic response to stress, or the peripheral response to the increased sympathetic activity. The observed attenuation of the HR response to stress and the altered reactivity to exogenous ANG support this concept; both have been reported by others (17). In contrast, an inhibition of the vascular response to NOR administration, which might be expected if GLA increased PGE production (18,19), was not observed in the present study.

In the present study GLA administration chronically attenuated the cardiovascular responses to an epigenetic stimulus, stress, without affecting the genetic predisposition to elevated BP, seen at rest. A previous study, conducted on the genetically hypertensive SHR, demonstrated that GLA supplementation, in the form of evening primrose oil, chronically reduced resting BP (20). The discrepancy in the results of the two studies probably is due to the early age of onset of GLA feeding in the SHR (5 weeks) relative to the SHR x WKY (10 weeks). It is possible that earlier onset of GLA administration in the SHR x WKY also will lower resting BP.

The effects of GLA on the stress responses of the SHR x WKY suggest that it may be effective in preventing the neurogenic triggering effect of chronic stress. If so, this could prevent the pathogenic changes accompanying the aggravation of the condition and reducing the risk of secondary complications of hypertension (3). To test this hypothesis, however, GLA administration must accompany a chronic stress situation sufficient to induce the neurogenic trigger.

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Dimers Formed in Oxygenated Methyl Linoleate Hydroperoxides

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ABSTRACT

The formation of dimers was demonstrated in methyl linoleate hydroperoxides decomposed by bubbling with dry air at 30 C. The dimer fraction isolated by gel permeation chromatography was further fractionated by successive silicic acid column and high performance liquid chromatography. Major components were analyzed after derivatizations by gas chromatography-mass spectrometry with several ionization methods, i.e., electron impact, chemical ionization and field desorption.

After aeration for 90 min, 1.4% of the hydroperoxides were decomposed. However, almost all of the secondary products were dimers, while polar monomeric and low molecular products were negligible. After aeration for 390 min, both polar monomeric (5.0%) and low molecular (0.8%) compounds formed, but dimers and polymers (18.1%) were still the major products. These results show the importance of polymerization in the aerobic breakdown of hydroperoxides.

The dimers isolated from hydroperoxides aerated 90 min could be separated into two fractions according to their polarities. The dimers identified usually were composed of octadecadienoate and oxygenated octadecenoate moieties crosslinked through either ether or peroxy linkages across C-9 or C-13 positions. The oxygen-containing functional groups found in the dimers include hydroperoxy, hydroxy and oxo groups. The polar dimers had two of these groups per molecule, while the less polar dimers had one. The main constituents of dimers were linked through peroxy bridges and found to be similar to the dimers previously identified in autoxidized methyl linoleate. These dimers are suggested as important intermediates in linoleate oxidation and as precursors of flavor deterioration.
Lipids 20:578-587, 1985.

INTRODUCTION

An early study (1) on the low-temperature decomposition of methyl linoleate hydroperoxides showed that products were primarily dimers, but the structural details were not determined. Two groups of workers also determined the structure of dimers in the anaerobically decomposed products of linoleic acid hydroperoxides (2,3) and methyl linoleate hydroperoxides (4). We believe, however, that the aerobic dimerization should be more important in the autoxidation mechanism of unsaturated esters.

The primary products of autoxidized lipids are monomeric hydroperoxides, which undergo further oxidation to produce a very complex mixture of volatile and non-volatile products (5,6). Among these secondary products, much attention has been given to volatile decomposition products because they cause a decrease in flavor quality and safety of foods (6). Nonvolatile monomeric oxygenated products formed by both auto- and photosensitized oxidation of linoleate and linolenate have been studied and elucidated extensively in the last decade (7-9). However, although early works with autoxidized linoleate and linolenate showed the formation of significant amounts of polar and polymeric materials (10-12), the detailed structures of these dimers and polymers were not established for a long time. In our previous studies (13-15), we found that hydroperoxides were accompanied by a considerable amount of dimers during the very early stage of autoxidation and elucidated the chemical structures of their main components.

In this paper, we describe the formation of dimers from methyl linoleate hydroperoxides decomposed in air and compare their structures with those formed in autoxidized methyl linoleate. The major constituents of dimers were isolated and analyzed spectrophotometrically and by gas chromatography-mass spectrometry (GC-MS).

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MATERIALS AND METHODS

Methyl Linoleate Hydroperoxides

Methyl linoleate was prepared from mixed methyl esters of safflower oil and purified by urea adduction, silicic acid column chromatography and vacuum distillation (13); it analyzed 99.8% by GC. Methyl linoleate was autoxidized at 30±1 C by bubbling with dry air for 10 days. The autoxidized methyl linoleate (280 g, peroxide value [PV]=1929) was subjected to silicic acid column chromatography (Wakogel C-100, 60x10cm; Wako Pure Chemical Industries,

Osaka, Japan). The column was eluted in steps with the following three solvents: (i) ether/hexane (5:95), 20 l; (ii) ether/hexane (20:80), 15 l; (iii) ether, 5 l. The hydroperoxide fraction eluted with ether/hexane (20:80) was further fractionated by dry column chromatography (Silica Woelm TSC, 160×3 cm; Woelm Pharma., Eschwege, West Germany) with ether/hexane (30:70). The eluents were monitored by thin layer chromatography (TLC), and the pure hydroperoxide gave a single spot on TLC. TLC conditions are described below. The purity of the hydroperoxide preparation was confirmed by PV measurement (6137 meq/kg, theoretical value:6135).

Percentage distribution of four isomeric hydroperoxides (*cis,trans-* and *trans,trans-* 9- and 13-hydroperoxy octadecadienoates) was determined by high performance liquid chromatography (HPLC) and calculated from the peak area of the corresponding hydroxy esters after reduction with $NaBH_4$ (16) on high performance liquid chromatography (HPLC) (17). HPLC was done on a Radial-Pak Cartridge Silica column (5μ, 10×0.8 cm; Waters Assoc., Milford, Massachusetts) using both a refractive index detector (Erma Optical Works, Tokyo, Japan) and a UV detector (UVIDEC-10011, Jasco Co., Tokyo, Japan) set at 233 nm for conjugated diene function. The solvent system was composed of isopropyl alcohol/hexane (0.7:99.3) and pumped at 2.0 ml/min. The isomeric composition calculated from HPLC was as follows: methyl 13-hydroxy-*cis*-9,*trans*-11-octadecadienoate: 30%, methyl 13-hydroxy-*trans*-9,*trans*-11-octadecadienoate: 20%, methyl 9-hydroxy-*trans*-10,*cis*-12-octadecadienoate: 32%, methyl 9-hydroxy-*trans*-10,*trans*-12-octadecadienoate: 18%. The proportions of 9- and 13-isomers are in accordance with the data in the literature (5).

Decomposition of Methyl Linoleate Hydroperoxides

Hydroperoxides (7 g) in a glass test tube were decomposed by bubbling with dry air through glass capillaries at 30±1 C for 390 min. Sample aliquots were analyzed for mean molecular weights (MMW) and by TLC at certain time intervals. MMW were determined by the vapor pressure equilibrium method using benzene on a Hitachi Perkin-Elmer Model 115 apparatus as described previously (13). TLC was carried out using Kieselgel 60 (precoated: 0.25 mm, Merck, Darmstadt, West Germany) plates and developed with a mixture of ether/hexane (40:60). Spots were visualized by spraying with 50% aqueous sulfuric acid and subsequent charring at 110 C for 20 min. For quantitative analysis, the charred chromatograms were scanned on a densitometer CS-900 (Shimadzu, Kyoto, Japan) using the transmittance mode with zig-zag scanning at 450 nm, and a Shimadzu Recorder U-225 MCS.

Gel Permeation Chromatography of Decomposed Hydroperoxides

After aeration for 90 and 390 min, the decomposed hydroperoxides were fractionated by gel permeation chromatography on a Bio-Beads S-X3 (Bio-Rad Laboratories, Richmond, California) column (180×2.7 cm) with benzene as the solvent. Eluents were monitored at 233 nm by a UV spectrometer (UVIDEC 320, Jasco, Tokyo, Japan). Mean molecular weight (MMW) was determined for each fraction by vapor pressure equilibrium as described previously (13). The monomer fraction obtained from samples aerated for 390 min was further fractionated on a silicic acid (Mallinckrodt Chemicals, St. Louis, Missouri) column (81.7×1.4 cm), and eluted in steps with ether/hexane (10:90) and ether to separate unreacted hydroperoxides from the secondary oxidation products.

Fractionation of Dimers

The dimeric fraction obtained by gel chromatography from hydroperoxides aerated for 90 min was separated first into two fractions (D_1, D_2) on a Sep-Pak Cartridge Silica (Waters Associates, Framingham, Massachusetts) and eluted with ether/hexane (2:8), 20 ml (D_2) and ether, 20 ml (D_1). Fractions D_1 and D_2 were further fractionated by HPLC into D_1-1,2 and D_2-1,2,3 respectively. HPLC was done under the same conditions as described above, except the solvent system consisted of isopropyl alcohol/hexane (2.5:97.5) for D_1.

Structural Investigation of Fractions D_1-1,2 and D_2-1,2,3

The intermolecular linkages were characterized by selective splitting as follows; Cleavage of C-O-O-C: The dimeric fraction was treated with $SnCl_2$ according to the method of Mizuno and Chipault (18). Cleavage of C-O-C: The dimeric fraction was reduced with hydroiodic acid by the method of Frankel et al. (12).

The hydroperoxy groups in dimers were specifically reduced with triphenylphosphine (Ph_3P) according to Neff et al. (8) and acetylated by dissolving in a mixture of acetic anhydride/pyridine (1:2, v/v), then allowed to stand overnight at room temperature.

Hydroxy groups in samples were silylated by bis trimethyl silyl acetamide (Tokyo Kasei, Tokyo, Japan).

A 1% solution of N,N′-dimethyl-p-phenylenediamine (DMPD) in a mixture of methanol/water/acetic acid (128:25:1) was sprayed on

TLC plates (19) to detect hydroperoxides as purple spots.

The UV spectrum was measured in a methanol solution on a Hitachi 124 (Hitachi, Tokyo, Japan) and the IR spectrum on a Jasco Model IR-S (Jasco, Tokyo, Japan).

The field desorption mass spectrum (FD-MS) was determined on a JMS 01-SG2 apparatus (Japan Electron Optics Lab, Tokyo, Japan) and chemical ionization mass spectrum (CI-MS) on a JMS-DX 300 (Japan Electron Optics Lab) using isobutane as the reagent gas. GC and GC-MS were done respectively on a Japan Electron Optics Lab 1100 (Japan Electron Optics Lab, Tokyo, Japan) and a Shimadzu LKB-9000 apparatus equipped with a glass column (200x 0.3 cm) containing 3% SE-30 on Chromosorb W (60/80 mesh), programmed from 200 to 240 at 4 C/min.

RESULTS

Decomposition of Methyl Linoleate Hydroperoxides

Before aeration, only a single spot of unreacted hydroperoxides with an Rf value 0.3 was detected by TLC. However, after 30 min, two tailing spots, D_1 (Rf 0.00-0.10) and D_2 (Rf 0.15-0.25), appeared and the percentage distribution of these polar materials estimated by a densitometer increased as the conjugated diene decreased. Therefore, the formation of polar materials accompanies the destruction of conjugated diene structures in hydroperoxides as observed previously (1,12,14,15,20).

MMW increased rapidly from the beginning (MMW:318) until 90 min (MMW:441), but then remained unchanged and finally decreased at 390 min (MMW:365). It is presumed, therefore, that polymerization is greater than fission in the initial stage of the decomposition of methyl linoleate hydroperoxides.

Methyl linoleate hydroperoxides aerated 90 min were separated by gel chromatography into two fractions (Fig. 1) with MMW 671 (Fr.-1) and 332 (Fr.-2), corresponding to dimers and monomers, respectively (theoretical value for monomer, 326). The polar materials in decomposed hydroperoxides were found exclusively in the dimer fraction (Fr.-1), and the monomer fraction gave a single spot corresponding to the original hydroperoxides on TLC. Therefore, it can be assumed that most of the secondary oxygenated materials formed in the initiation stage of hydroperoxide degradation was dimeric; this accounts for the rapid increase of MMW in the early stage of aeration.

The percentage composition of four isomeric hydroperoxides in the monomer fraction (Fr.-2) were almost the same as before aeration. This result suggests either that the tendency for dimerization is equal for all isomers or that unpolymerized hydroperoxides have been isomerized and reached equilibrium during incubation (21).

With the progress of decomposition, the gel chromatogram became more complex as shown in Figure 2, indicating the formation of polymers (T) and low molecular weight products (L_1, L_2) in addition to monomer (M) and dimer (D). As 95% of the monomer fraction was composed of unreacted hydroperoxides, the dimer fraction was still the major component of the secondary oxidation products, while the low

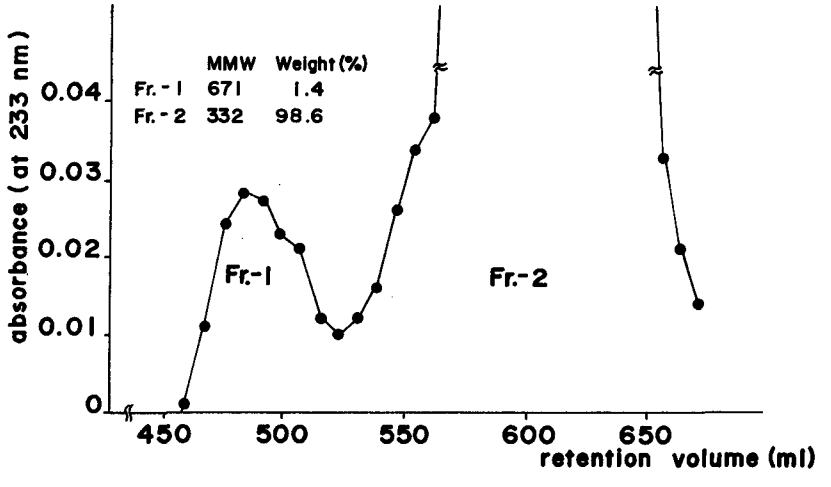


FIG. 1. Fractionation of methyl linoleate hydroperoxides aerated 90 min on a Bio-Beads S-X3 column.

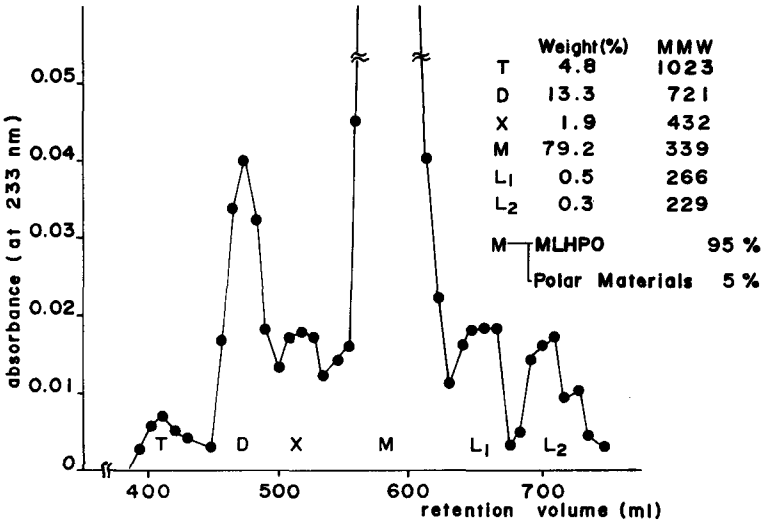


FIG. 2. Fractionation of methyl linoleate hydroperoxides aerated 390 min on a Bio-Beads S-X3 column.

molecular products were found in amounts of less than 1%. This result indicates the importance of polymerization in the decomposition of hydroperoxides.

Structures of Dimers

Figure 3 shows the HPLC patterns of D_1 and D_2 dimers isolated from hydroperoxides aerated 90 min by successive gel and silicic acid column chromatography. By preparative HPLC, two (D_1-1,2) and three (D_2-1,2,3) major subfractions were isolated to determine their structures.

Fraction D_1-1

This fraction was not depolymerized with $SnCl_2$, but treatment with hydroiodic acid cut MMW in half. Therefore, D_1-1 is linked through a C-O-C bond.

The UV spectrum showed an absorption at 233 nm derived from conjugated diene. The IR spectrum showed the presence of OH (or OOH) (3360 cm^{-1}) and α,β-unsaturated oxo groups (1630, 1660 and 1670 cm^{-1}). The absorption at 3360 cm^{-1} could be assigned to OH from the negative coloring with DMPD and from FD-MS.

FD-MS gave a molecular weight of 634, corresponding to two moles of linoleate plus three atoms of oxygen, by the molecular ion peak together with the characteristic peaks at m/e 619 (M-CH_3) and 617 (M-OH) (Fig. 4).

CI-MS data in Table 1 also supported the molecular weight (634) by the prominent peaks at m/e 635 (M+H), 634 (M), 617 (MH-H_2O) and 603 (MH-CH_3OH). The fragment peaks at

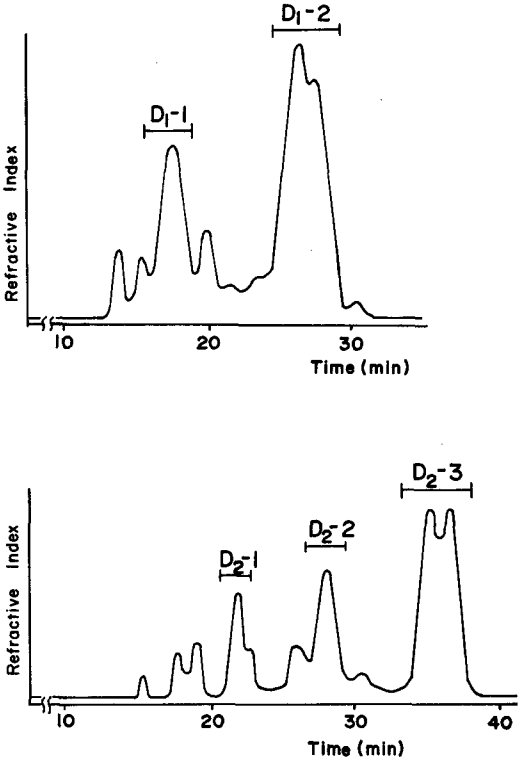


FIG. 3. HPLC of D_1 and D_2 dimeric fractions. Flow rate, 2 ml/min. Mobile phase, 2.5% (for D_1) and 0.7% (for D_2) isopropyl alcohol in hexane.

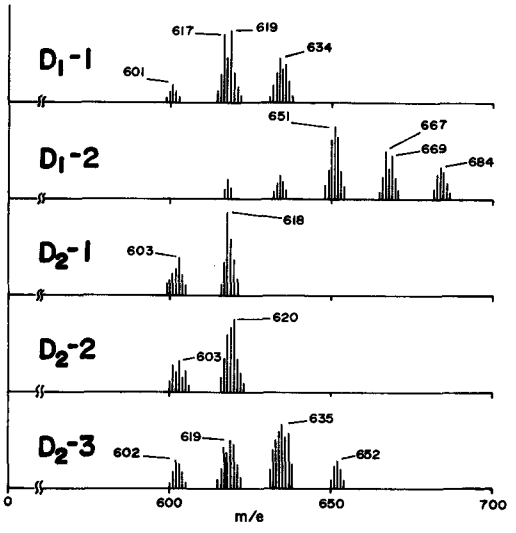
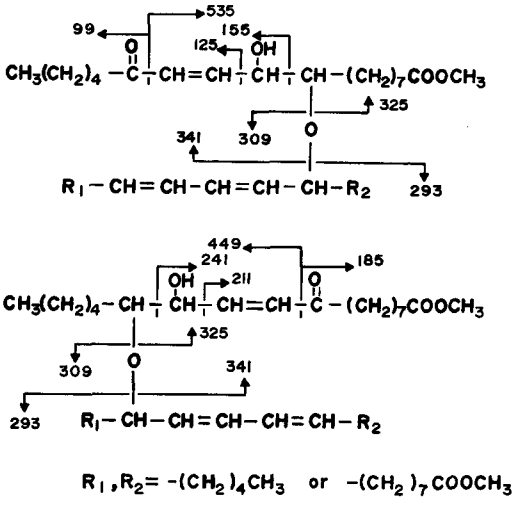


FIG. 4. FD-MS of fractions D_1-1,2 and D_2-1,2,3.



SCHEME 1

m/e 535 (M-99), 99 or 449 (M-185), 185 indicated the presence of an oxo group on C-9 or C-13, respectively (Scheme 1). The peaks at 155, 125 or 423 (M-211), 241, 211 showed the position of a hydroxy group on C-10 or C-12 respectively (Scheme 1). The fragment peaks at m/e 341, 325, 309 and 293 should be derived from the cleavage across the ether linkage. These data suggest that fraction D_1-1 is a dimer of methyl octadecadienoate and an isomeric mixture of 9-oxo-12-hydroxy- and 13-oxo-10-hydroxy-octadecadienoate linked through an ether bond, as shown in Scheme 1. The other

TABLE 1

CI- and GC-MS Data

CI-MS data of HPLC fraction D_1-1

Observed mass	Relative intensity	Observed mass	Relative intensity
635 (MH)	1.8	239	68.8
634 (M)	0.3	211	25.0
633 (M-1)	1.5	209	16.5
617 (MH-H_2O)	2.3	187	100.0
603 (MH-CH_3OH)	1.4	185	31.5
535 (M-99)	1.6	157	56.5
449 (M-185)	4.1	155	59.0
423 (M-211)	1.7	153	66.7
341	8.3	129	26.0
325	37.5	125	10.0
309	100.0	123	12.0
293	56.5	101	59.0
241	19.0	99	59.0

Characteristic ions of EI-MS of GC fraction P-4

Observed mass	Relative intensity	Observed mass	Relative intensity
485 (M-CH_3)	1.0	271 (M-229)	17.6
469 (M-OCH_3)	1.4	259	100.0
410 (M-HOTMS)	1.2	245	19.1
357 (M-143)	4.3	229	26.2
331	5.7	173	91.7
		143	29.5

CI-MS data of HPLC fraction D_2-1

Observed mass	Relative intensity	Observed mass	Relative intensity
619 (MH)	32.7	293	68.4
618 (M)	10.7	239	67.2
617 (M-1)	25.1	225	22.5
587 (MH-CH_3OH)	3.4	209	15.4
519 (M-99)	4.8	187	94.4
479 (M-139)	1.8	185	22.6
433 (M-185)	5.6	139	15.4
393 (M-225)	2.1	123	11.5
325	14.1	101	26.7
309	100.0	99	19.2

CI-MS data of HPLC fraction D_2-2

Observed mass	Relative intensity	Observed mass	Relative intensity
621 (MH)	14.5	311	59.6
620 (M)	4.7	309	100.0
619 (M-1)	13.3	293	100.0
603 (MH-H_2O)	6.6	239	90.7
601 (M-1-H_2O)	10.4	227	26.1
589 (MH-CH_3OH)	10.1	187	100.0
519 (M-101)	21.5	153	94.8
433 (M-187)	22.0	141	15.4
327	33.4	101	42.6
		99	30.0

fragment peaks at m/e 239 (HO-CH-CH=CH-CH=CH-$[CH_2]_7COOCH_3$), 209 (CH=CH-CH=CH-$[CH_2]_7COOCH_3$), 187 (HO-CH-$[CH_2]_7COOCH_3$), 153 ($CH_3[CH_2]_4CH$=CH-CH=CH-CH-OH), 123 ($CH_3[CH_2]_4CH$=CH-CH=CH), 101 ($CH_3[CH_2]_4CH$-OH) indicate the presence of an ether linkage between a pair of C_{18} esters across C-9 and/or C-13 positions. Although the peaks at 198 or 101 could be derived from the cleavage between C_{10}-C_9 (OH) or C_{13} (OH)-C_{12} positions respectively, this possibility was not supported by the experimental evidence based on CI-MS after silylation, showing no fragment peaks at m/e 259 (TMSOCH$[CH_2]_7COOCH_3$) or 173 ($CH_3[CH_2]_4$CHOTMS). The presence of masses 313 (TMSOCH-CH=CH-C[=O]-$[CH_2]_7COOCH_3$) and 227 ($CH_3[CH_2]_4$-C[=O]-CH=CH-CHOTMS) supported the structure described above.

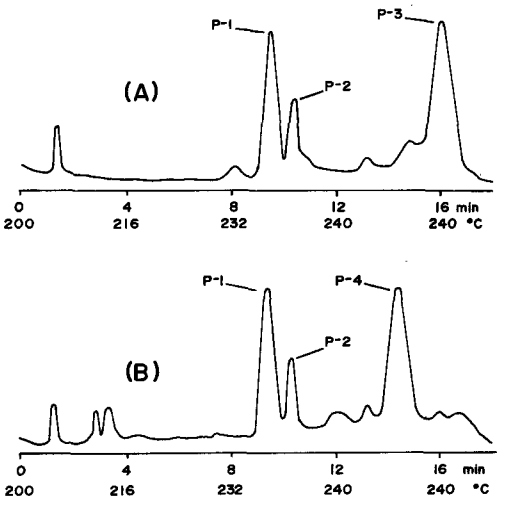


FIG. 5. Gas chromatogram of D_1-2. (A) Reduced with $SnCl_2$ and silylated. (B) Reduced with $SnCl_2$ and silylated after reduction with Ph_3P and acetylation. 3% SE-30 on Chromosorb W packing in a 200×0.3 cm column, 200-240 C at 4 C/min temperature program.

Fraction D_1-2

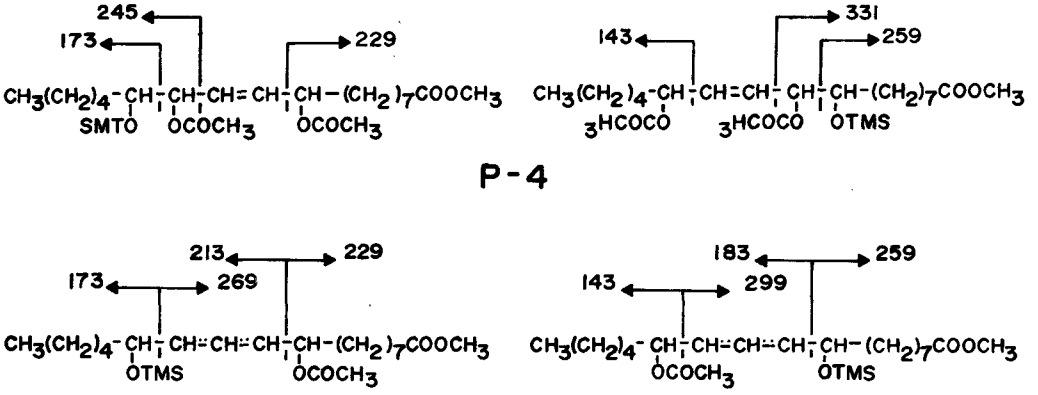
By the reduction with $SnCl_2$, fraction D_1-2 was depolymerized and gave a MMW of 332. This result showed that dimer D_1-2 was linked through C-O-O-C bonds.

The reaction of D_1-2 with DMPD yielded a purple spot on TLC, suggesting the presence of hydroperoxy group(s), which is supported by the IR spectrum showing strong absorption at 3450 cm^{-1}. The UV spectrum showed the presence of conjugated diene with an absorption maximum at 233 nm.

The peak at m/e 684 (M) on the FD-MS spectrum, together with other masses at m/e 669 (M-CH_3), 667 (M-OH) and 651 (M-OOH), suggested a molecular weight of 684, corresponding to two moles of linoleate plus six atoms of oxygen (Fig. 4).

Figure 5-A shows GC of dimer fraction D_1-2 after reduction with $SnCl_2$ and silylation. Three major peaks, P-1, -2 and -3, were analyzed by GC-MS. The EI-mass spectra were almost identical for peaks P-1 and P-2, and indicated TMS derivatives of an isomeric mixture of methyl 9- and 13-hydroxy-octadecadienoate from the following masses: 382 (M:19.3[P-1],30.8[P-2], relative intensity), 311 (M-$CH_3[CH_2]_4$:32.6 [P-1],57.7[P-2]), 292 (M-HOTMS:36.6[P-1], 23.1[P-2]), 259 (TMSOCH$[CH_2]_7COOCH_3$: 26.4[P-1],5.8[P-2]), 225 (M-$[CH_2]_7COOCH_3$: 100.0[P-1,2]) and 173 (TMSOCH$[CH_2]_4CH_3$: 35.6[P-1],9.6[P-2]). P-1 and P-2 were expected to be TMS ethers of *trans,cis* and *trans,trans* OH isomers, respectively (22). P-3 gave an EI-mass spectrum similar to that reported by Terao et al. (20) for 9,10,13- and/or 9,12,13-triOTMS-octadecenoate. Major fragment peaks of P-3 were as follows: 545 (M-CH_3:0.2, relative intensity), 470 (M-HOTMS:1.7), 460 (M-$CH_3[CH_2]_4CHO$: 10.6), 387 (M-TMSOCH$[CH_2]_4CH_3$:4.6), 301 (M-301 M-TMSOCH$[CH_2]_7COOCH_3$:16.7), 259 (TMSOCH$[CH_2]_7COOCH_3$:60.1), 173 (TMSOCH$[CH_2]_4CH_3$:100.0). As the total GC peak area of monohydroxy esters (P-1 + P-2) was almost equal to that of the trihydroxy ester (P-3), we assumed that one mole of monohydroxy and one mole of trihydroxy esters are generated by the cleavage with $SnCl_2$ of a peroxide linkage in a D_1-2 dimer molecule. Since $SnCl_2$ reduces hydroperoxy groups or peroxide linkages but does not reduce oxo groups (16), GC-, FD-MS and other results show that D_1-2 is a dimer of an octadecadienoate ester and a dihydroxy C_{18} ester linked through a peroxy linkage.

The site of the peroxide linkage in the trihydroxy esters derived from two hydroperoxy and a peroxide linkage was determined by GC-MS (Fig. 5-B) after the successive reduction of hydroperoxy groups with Ph_3P and acetylation prior to the breakdown of the peroxide linkages, as described previously (15). The major peaks in the EI-mass spectrum of the diacetoxy mono-OTMS C_{18} esters (Table 1) were almost equal to those isolated from the dimers formed in autoxidized methyl linoleate (15) and supported the fragmentation shown in Scheme 2. Therefore, the occurrence of OTMS groups was suggested on C-9 or C-13 positions. This result indicates that D_1-2 dimers may be linked at C-9 or C-13 positions rather than at C-10 or C-12 positions.



SCHEME 2

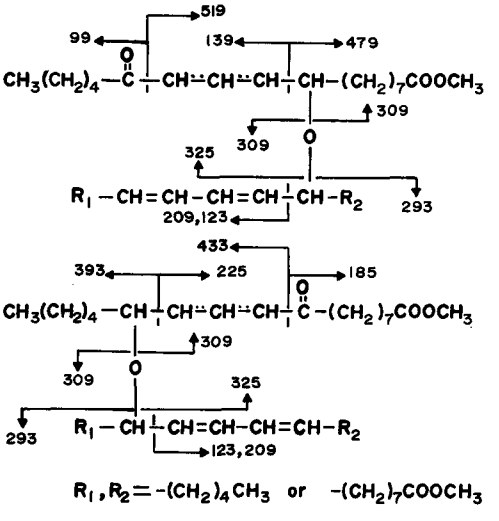
Fraction D_2-1

The intermolecular linkage was not cleaved with $SnCl_2$, but with HI, thus indicating that dimers D_2-1 are linked through C-O-C bonds.

The UV absorption showed a maximum at 233 nm for conjugated diene. The IR spectrum indicated an absorption at 1630, 1660 and 1700 cm^{-1} due to α,β-unsaturated oxo groups, and neither OH nor OOH absorption was detected.

FD-MS of fraction D_2-1 (Fig. 4) indicated a molecular weight of 618 by the distinct peaks at m/e 618 (M) and 603 (M-CH_3), which corresponds to two moles of linoleate plus two atoms of oxygen.

CI-MS data in Table 1 show prominent peaks at m/e 619 (MH), 618 (M), 617 (M-1) and 587 (MH-OCH_3) supporting the molecular weight determined by FD-MS. From the molecular weight and the chemical characteristics, D_2-1 is suggested to be a dimer of an oxo octadecenoate and an octadecadienoate ester linked through an ether linkage. The fragmentation shown in Scheme 3 together with the following peaks supported a postulated structure in which an isomeric mixture of 9-and 13-oxooctadecenoate and an octadecadienoate ester are linked across C-9 or C-13 positions through an ether bond: 239 (HO-CH-CH=CH-CH=CH-[CH_2]$_7$$COOCH_3$), 187 (HO-CH-[$CH_2$]$_7$$COOCH_3$), 123 ($CH_3$[$CH_2$]$_4$-CH=CH-CH=CH), 101 ($CH_3$[$CH_2$]$_4$CH-OH). The IR spectrum suggested the occurrence of α,β-conjugated oxo groups (1630, 1660 and 1670 cm^{-1}). However, there is the possibility that D_2-1 consisted of an isomeric mixture with the double bond at C-10 and C-11.



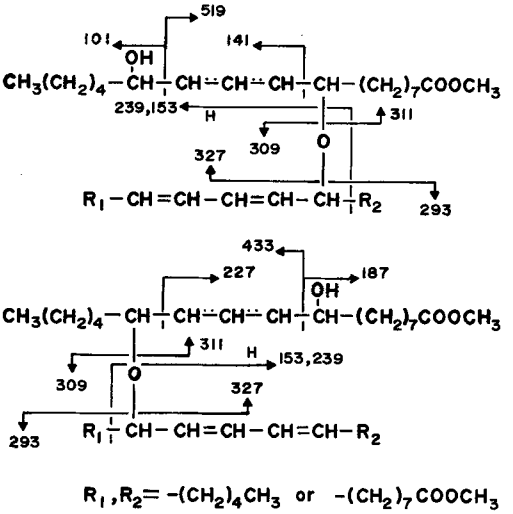
SCHEME 3

Fraction D_2-2

The intermolecular C-O-C linkage of D_2-2 was shown by the fact that $SnCl_2$ did not cause cleavage, whereas HI did. UV and IR analyses indicated the presence of conjugated diene and OH or OOH by absorptions at 233 nm and 3360 cm^{-1}, respectively. The latter was identified as OH from the molecular weight and the negative coloring reaction with DMPD.

FD-MS (Fig. 4) gave a molecular weight 620, corresponding to two moles of linoleate plus two atoms of oxygen with the peaks at 620 (M) and 603 (M-OH).

CI-MS showed the characteristic peaks which also agreed with the molecular weight of 620 (Table 1). The fragmentation pattern by CI-MS suggested structures in which an isomeric mixture of octadecenoate esters with a hydroxy group on C-9 or C-13 is linked with an octadecadienoate through an ether linkage as shown in Scheme 4. The position of the hydroxy group was determined by CI-MS of silylated D_2-2 showing distinct peaks at m/e 259 (TMSOCH $[CH_2]_7COOCH_3$) and 173 ($CH_3[CH_2]_4$CHOT MS). However, the exact location of the double bond (C-10 or C-11) could not be determined by MS.



SCHEME 4

Fraction D_2-3

This fraction was depolymerized with $SnCl_2$, indicating that it is linked through a C-O-O-C bond. The purple coloring with DMPD and IR absorption at 3600 cm^{-1} indicated the presence of hydroperoxy group(s). The UV spectrum showed the presence of conjugated diene with an absorption maximum at 233 nm.

FD-MS (Fig. 4) gave a distinct molecular ion peak (652) corresponding to two moles of linoleate plus four atoms of oxygen with the fragment peaks at m/e 635, 619 and 602, which are presumed to be derived from M-OH, M-OOH and M-(OOH+OH), respectively.

After reduction with $SnCl_2$ and silylation, D_2-3 gave three peaks with different retention times on GC (Fig. 6-C). The EI-mass spectra were almost identical between peaks P-1 and P-2, and indicated that they were TMS derivatives of methyl 9- and/or 13-hydroxy-octadecadienoate by the characteristic peaks (382 [M], 311, 259, 225, 173) (21). P-5 was identified as the TMS derivative of methyl 9,13-dihydroxy octadecenoate by the following EI-mass spectral data: 472 (M:1.1, relative intensity), 457 (M-CH_3:3.4), 441 (M-OCH_3:3.4), 382 (M-HOTMS:6.7), 315 (M-$[CH_2]_7COOCH_3$:7.0), 299 (M-173:49.2), 259 (TMSOCH$[CH_2]_7$ $COOCH_3$:79.8), 213 (M-259:25.3) and 173 ($CH_3[CH_2]_4$CHOTMS:100.0) (7,20). Total peak area of the monohydroxy esters was approximately equal to that of dihydroxy esters on GC. Therefore, the dimer consists of one mole of monohydroxy and one mole of dihydroxy esters linked by a peroxide bond. It is also presumed that D_2-3 contained one hydroperoxy group per molecule. We tried to determine whether hydroxy group on (C-9 or C-13) in octadecenoate is responsible for the peroxide linkage by GC-MS after selective reduction with Ph_3P followed by acetylation and silylation as described for D_1-2 (Fig. 6-D). EI-mass spectra showed that D_2-3 is an isomeric mixture with a peroxide linkage across either C-9 or C-13 of a hydroperoxy octadecenoate ester and an octadecadienoate ester as shown in Scheme 2 (P-6). The double bond would be located between C:10-11 or C:11-12.

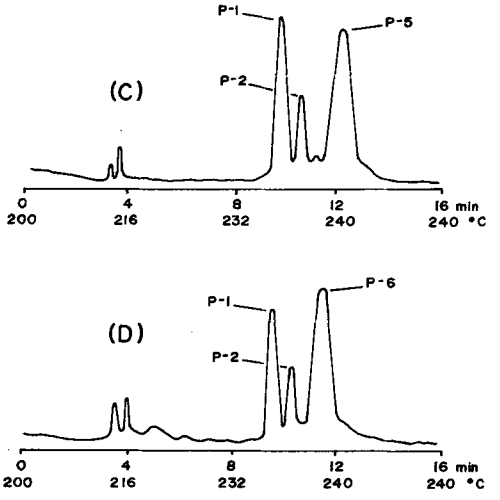


FIG. 6. Gas chromatogram of D_2-3. (C) Reduced with $SnCl_2$ and silylated. (D) Reduced with $SnCl_2$ and silylated after reduction with Ph_3P and acetylation. Experimental conditions were the same as in Figure 5.

All the dimer structures and relative concentrations determined in this study are summarized in Figure 7. D_1-2 and D_2-3 were identified previously as products of autoxidized methyl linoleate (14,15).

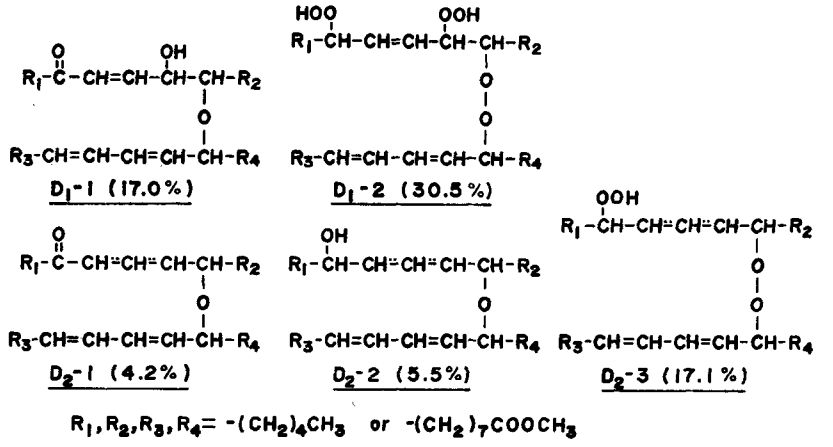


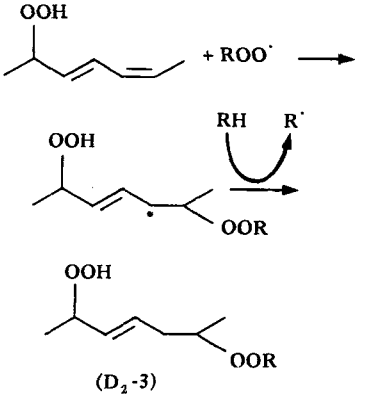
FIG. 7. Proposed structures of D_1-1,2 and D_2-1,2,3.

DISCUSSION

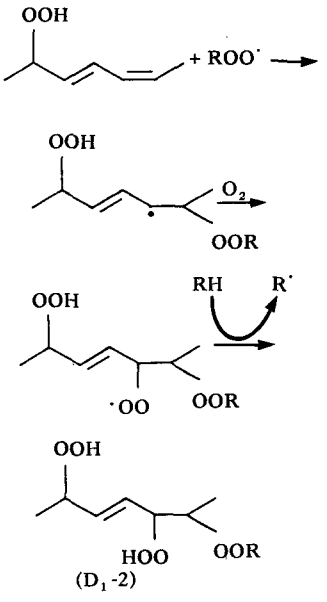
This investigation shows that the first major products formed by aerobic decomposition of methyl linoleate hydroperoxides are dimers, and that there is a lag in the appearance of other secondary products such as bifunctional oxygenated monomers and low molecular fission products. This observation also was made during the autoxidation of methyl linoleate (13). The generation of hydroperoxyl and alkyl radicals from lipid hydroperoxides without addition of metals already has been suggested by the exchange of oxygen between hydroperoxy groups and the atmosphere (21) and the geometric isomerization of hydroperoxides (23). Therefore, it seems reasonable that the termination of these radicals, which results in the formation of dimers, precedes other degradative reactions.

Some workers decomposed linoleic acid hydroperoxides in dilute solutions in the presence of transition metals (3,24) or radicals (2). Under these conditions, hydroperoxides generally decomposed to produce various oxygenated monomers and fission products but not polar dimers. In their experiments, dimers were yielded only when a large quantity of metals was added (24) or when the atmosphere was replaced by nitrogen (2). A large quantity of metals should enhance the concentration of radicals in the reaction mixtures, and a nitrogen atmosphere would prevent scavenging of radicals by O_2 in the reaction mixtures. Consequently, in dilute solutions containing metals, the radicals generated by the breakdown of hydroperoxides, which are supposed to be mainly alkoxy radicals, prefer to form various oxygenated monomers rather than dimerize because of their low concentration and short lives.

As summarized in Figure 7, the dimers found in the present investigation are linked through either C-O-O-C or C-O-C bonds. These structures are quite different from those of the dimers yielded in the thermally decomposed methyl linoleate hydroperoxides, which are linked through C-C bonds (12). Therefore, the C-O-O-C and C-O-C linkages should be characteristic of polymerization carried out under the mild conditions. The dimers with C-O-O-C linkages (D_1-2 and D_2-3) were the main components and also were isolated from the autoxidized methyl linoleate in the very early stages (PV: 16-22) (14,15). The fact that the dimers found in autoxidized methyl linoleate also could be induced from methyl linoleate hydroperoxides suggest a pathway of dimer formation during autoxidation via hydroperoxides. D_2-3 could originate from intermolecular addition of hydroperoxides with peroxy radicals as follows:



D_1-2 would transform from D_2-3 by further oxygenation (15) or form de novo via the common intermediate radicals with D_2-3 as follows:



(D_1-2)

The dimers linked through C-O-C bonds would be formed by intermolecular addition of an alkoxy radical to a double bond. These dimers especially linked through -C-O-O-C- are very labile and easily broken to form numerous compounds including low molecular fission products (25). Consequently, it is reasonable that these dimers are important intermediates of linoleate autoxidation causing flavor deterioration.

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Characterization of Gangliosides of Porcine Erythrocyte Membranes: Occurrence of Ganglioside G_{D3} as Major Ganglioside

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ABSTRACT

Four major ganglioside species were isolated from porcine erythrocyte membranes by DEAE-Sephadex and Iatrobeads column chromatography. Treatment of the lipids with graded neuraminidase and β-galactosidase, gas chromatographic analysis of their carboyhydrates, sphingosine bases and molecular species of sialic acid revealed that the structure of these gangliosides were G_{M3}(NeuAc), G_{M3}(NeuGc), G_{D3}(NeuAc) and G_{D3}(NeuGc), each of which was 16 ± 2 μg, 304 ± 42 μg, 30 ± 3 μg and 240 ± 26 μg, respectively, per gram of the dry erythrocyte stroma. The amount of G_{M3} and G_{D3} accounted for more than 95% of total gangliosides of the erythrocytes. Porcine erythrocytes may provide a good source for large scale preparation of ganglioside G_{D3} which recently was identified as a human melanoma-associated antigen.
Lipids 20:588-593, 1985.

INTRODUCTION

Chemical structures of major neutral glycosphingolipids of porcine erythrocytes, which previously had been shown to be the globoside type (3), were determined by Miyatake, Handa and Yamakawa (4). The metabolic relation of neutral glycosphingolipids between porcine plasma and erythrocytes also has been studied (5). However, the occurrence of gangliosides in porcine erythrocytes has been demonstrated so far only; the early reports of Yamakawa and Suzuki (6) and Klenk and Lauenstein (7). In the present paper, major gangliosides of porcine erythrocytes were isolated and their contents and structures characterized. We found that a large part (ca. 39%) of porcine erythrocyte gangliosides was ganglioside G_{D3}(NeuGc).

MATERIALS AND METHODS

Glycosphingolipids were extracted (8) from dry porcine erythrocyte stroma prepared according to the method of Yamakawa and Suzuki (6). The alkaline-stable lipids were loaded on a DEAE Sephadex A-25 column (acetate form) (9), from which mono- and disialoglycosphingolipids were separated by eluting with ammonium acetate-methanol in a linear gradient concentration system (0-0.5 M) (9). These gangliosides were further purified by repeating Iatrobeads 6RS-8060 (Iatron Lab. Inc., Tokyo) column chromatography (9,10). Monosialo- and disialogangliosides with *N*-acetyl- or *N*-glycolylneuraminic acid were separated by a linear gradient concentration system of $CHCl_3/CH_3OH$/2.5 N ammonia (75:23:1-30:70:4, v/v/v). Purified monosialo- and disialogangliosides were designated as ganglioside I and II, respectively. Gangliosides and neutral glycosphingolipids were analyzed by TLC on a silica gel 60 (Merck) with the following solvent systems: $CHCl_3/CH_3OH/H_2O$ containing 0.02% $CaCl_2 \cdot 2H_2O$ (60:40:9, v/v/v, solvent 1), $CHCl_3/CH_3OH$/2.5 N ammonia (60:35:8, v/v/v, solvent 2) and $CHCl_3/CH_3OH/H_2O$ (60:25:4, v/v/v, solvent 3), and visualized by spraying with resorcinol-HCl (11) and α-naphthol-H_2SO_4 (12) reagent, respectively. Individual gangliosides also were determined by scanning densitometry (13).

Gas liquid chromatographic (GLC) analysis of methyl esters of fatty acids and trimethylsilyl derivatives of methyl glycosides was performed with a column of 10% Silar 10 C on Gas-Chrom Q and 3% SE-30 on Simalite W, respectively. Sphingosine was analyzed according to the method of Ando and Yu (14). *N*-acetyl- and *N*-glycolylneuraminic acid were quantified by the method of Yu and Ledeen

*To whom correspondence should be addressed.

Abbreviations and nomenclature: NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; GlcCer, Glcβ1-lceramide; LacCer, lactosylceramide, Galβ1-4Glcβ1-lceramide; CTH, $GbOse_3Cer$, Galα1-4Galβ1-4Glcβ1-lceramide; G_{M3}(NeuAc), II^3NeuAcLacCer, NeuAcα2-3Galβ1-4Glcβ1-lceramide; G_{M3}(NeuGc), II^3NeuGcLacCer, NeuGcα2-3Galβ1-4Glcβ1-lceramide; G_{D3}(NeuAc), II^3(NeuAc)$_2$ LacCer, NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ1-lceramide; G_{D3}(NeuGc), II^3(NeuGc)$_2$ LacCer, NeuGcα2-8NeuGcα2-3Galβ1-4Glcβ1-lceramide. Gangliosides are named according to Svennerholm (1) and the recommendation of the IUPAC-IUB Commission on Biochemical Nomenclature (2).

(15). Purified gangliosides were treated with neuraminidase of *Cl. perfringens* (Sigma type VI) or *A. ureafaciens* (Nakarai Chem. Inc., Kyoto) (16) and β-galactosidase of *C. lampas* (Seikagaku kogyo Co. Ltd., Tokyo) (17).

RESULTS AND DISCUSSION

Two sialoglycolipids (gangliosides I and II) of porcine erythrocyte membranes were separated by chromatography on a DEAE-Sephadex column and further purified by Iatrobeads column. Final yields of the purified gangliosides from 150 g of lyophilized stroma were as follows: Ganglioside I, 48 mg (0.32 mg/g dry stroma); Ganglioside II 40 mg (0.27 mg/g dry stroma). Densitometric analysis of the spots on thin layer chromatogram of the total gangliosides showed the molar ratio of sialic acid in Ganglioside I to Ganglioside II was 0.8, and both gangliosides accounted for about 95.7% of the total gangliosides of the erythrocytes. Final preparations of gangliosides I and II migrated on a silica gel thin layer plate to the positions with nearly the same R_f values as G_{M3} and G_{D3}, respectively, in the neutral solvent 1 (Fig. 1A). Gangliosides I and II were separated to their subfractions, a fast-moving (Ganglioside I-a, II-a) and a slow-moving component (Ganglioside I-b, II-b) in the solvent 2 containing ammonia (Fig. 1B). Gangliosides I-a and -b have the same R_f values as G_{M3} (NeuAc) (human liver [18] and G_{M3}(NeuGc) (bovine erythrocyles [8,19]), respectively. Gangliosides II-a and II-b were isolated by Iatrobeads column chromatography (Fig. 1-C). Molar ratios of sialic acid/Gal/Glc/long chain base of Ganglioside I, II-a and II-b were about 1:1:1:1, 2:1:1:1 and 2:1:1:1, respectively (Table 1). Long chain base consisted mainly of $C_{18:1}$ sphingosine. Species of neuraminic acid in Gangliosides II-a and II-b were identified as *N*-acetyl- and *N*-glycolylneuraminic acid, respectively. A striking difference in fatty acid composition of Gangliosides I and II was found (Table 2). The fatty acid of $C_{24:0}$ was identified as a major component of Ganglioside I, whereas the higher content of the acid in Ganglioside II was shown by the acid of $C_{16:0}$, and only a very low content of $C_{24:0}$ fatty acid was detected.

Hydrolysis of Ganglioside I with neuraminidase *(Cl. perfringens)* gave a spot with the same R_f as LacCer on thin layer plate (Fig. 2). Treatment of 0.2 mg of the desialylated derivative of Ganglioside I with 0.01 unit of *C. lampas* β-galactosidase for 15 hr resulted in the formation of GlcCer which was shown by TLC (Fig. 2). Accordingly, since the neuraminic acid of Ganglioside I was composed of NeuGc and NeuAc type (Table 1) and Gangliosides I-a and I-b were comigrated with standard G_{M3}(NeuAc) and G_{M3}(NeuGc) (Fig. 1), the structure of

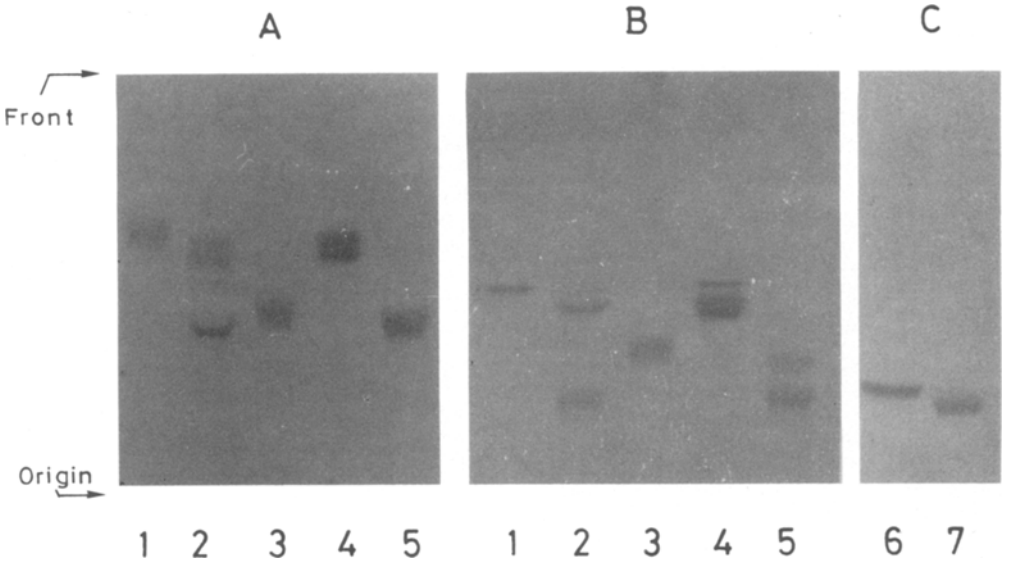

FIG. 1. Thin layer chromatogram of purified Gangliosides I and II. 1, G_{M3}(NeuAc); 2, total gangliosides of porcine erythrocytes; 3, G_{D3}(NeuAc); 4, Ganglioside I; 5, Ganglioside II; 6, Ganglioside II-a; 7, Ganglioside II-b. Plate A was developed with solvent 1 ($CHCl_3/CH_3OH/H_2O$ containing 0.02% $CaCl_2 \cdot 2H_2O$ [60:40:9, v/v/v]), and plates B and C with solvent 2 ($CHCl_3/CH_3OH$/2.5 N NH_3 (60:35:8, v/v/v). Bands were visualized with resorcinol-HCl reagent.

TABLE 1

Carbohydrate and Long Chain Base Composition of Ganglioside I, Ganglioside II-a and II-b in Porcine Erythrocyte Membranes

	Ganglioside I	Ganglioside II-a	Ganglioside II-b
Molar ratio of carbohydrate and long chain base			
Glucose	1.00	1.00	1.00
Galactose	0.95	0.96	0.97
Sialic acid	1.03	1.92	1.80
Long chain base	0.91	0.91	0.95
Sialic acid composition (%)			
N-acetylneuraminic acid	95.4	100	N.D.[a]
N-glycolylneuraminic acid	4.6	N.D.[a]	100
Long chain base (sphingosine) composition (%)			
$C_{18:1}$	92.0	91.5	90.1
Others	8.0	8.5	9.9

Neutral sugars were analyzed by GLC as trimethylsilyl derivatives of methylglycosides on 3% SE-30 column programmed from 140 C to 220 C at 2 C/min. Sialic acid species and sphingosine were analyzed by GLC as described in (15) and (14), respectively.

[a]Not determined.

TABLE 2

Fatty Acid Composition of Gangliosides I and II in Porcine Erythrocyte Membranes

Fatty acids	Ganglioside I	Ganglioside II
$C_{14:0}$	1.0	4.4
$C_{14:1}$	0.8	3.8
$C_{16:0}$	13.2	36.4
$C_{16:1}$	2.8	10.4
$C_{18:0}$	7.4	10.1
$C_{18:1}$	6.2	21.5
$C_{20:0}$	3.4	2.2
$C_{20:1}$	0.6	tr[a]
$C_{22:0}$	15.4	4.0
$C_{22:1}$	1.2	1.8
$C_{24:0}$	44.6	3.6
$C_{24:1}$	3.4	1.8

The values are given as weight percent. GLC of fatty acid methyl esters was carried out on a 10% Silar 10 C column programmed from 170 C to 250 C at 2 C/min.

[a]Trace (less than 0.4%).

Gangliosides I-a and I-b was confirmed to be G_{M3}(NeuAc) (5 ± 0.5% of total G_{M3}, 16 ± 2 μg/g dry stroma) and G_{M3}(NeuGc) (95 ± 3% of total G_{M3}, 304 ± 42 μg/g dry stroma), respectively. Brief incubation of Gangliosides II-a and II-b with 0.05 unit of *Cl. perfringens* neuraminidase for 20 min at room temperature around 20 C gave products which still contained sialic acid migrating to the same positions as G_{M3}(NeuAc) (human liver) and G_{M3} (NeuGc) (bovine erythrocytes) on a silica gel thin layer plate in solvent 2, respectively (Fig. 3). The mixture of Ganglioside II-a and II-b was converted completely to a glycolipid showing the same R_f as LacCer on TLC by the treatment with 0.1 unit of neuraminidase *(Cl. perfringens* or *A. ureafaciens)* for 4 hr at 37 C. Incubation of the reaction product thus obtained with β-galactosidase (0.1 unit, *C. lampas*) resulted in the formation of GlcCer (Fig. 4). GLC analysis of sialic acid species showed that Gangliosides II-a and II-b contained only NeuAc and NeuGc, respectively (Table 1). Gangliosides II-a and II-b have the same R_f value on silica gel thin layer plate in solvent 1 and 2 as standard G_{D3}(NeuAc) (NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ1-lceramide) from porcine kidney (17) (a gift from Dr. Ishizuka, Teikyo University) and G_{D3}(NeuGc) (NeuGcα2-8NeuGcα2-3Galβ1-4Glcβ1-lceramide) from bovine liver (20) (data not shown). The above results indicated that the structure of Ganglioside II-a was G_{D3}(NeuAc) (11 ± 0.5% of total G_{D3}, 30 ± 3 μg/g dry stroma) and that of Ganglioside II-b was G_{D3}(NeuGc) (89 ± 1.5% of total G_{D3}, 240 ± 26 μg/g dry stroma). Structures and the contents of major gangliosides of porcine erythrocytes are summarized in Table 3.

G_{D3} gangliosides have been reported in bovine liver (20), spleen (20), kidney (20, thyroid (21) and adrenal medulla (22) and also cat erythrocytes (23). This is the first report on the occurrence of G_{D3}(NeuGc) as major ganglioside (ca. 39% of the total porcine erythrocyte gangliosides) as well as the characterization of the gangliosides in porcine erythrocytes. Receptor activities of G_{M3} and G_{D3} gangliosides

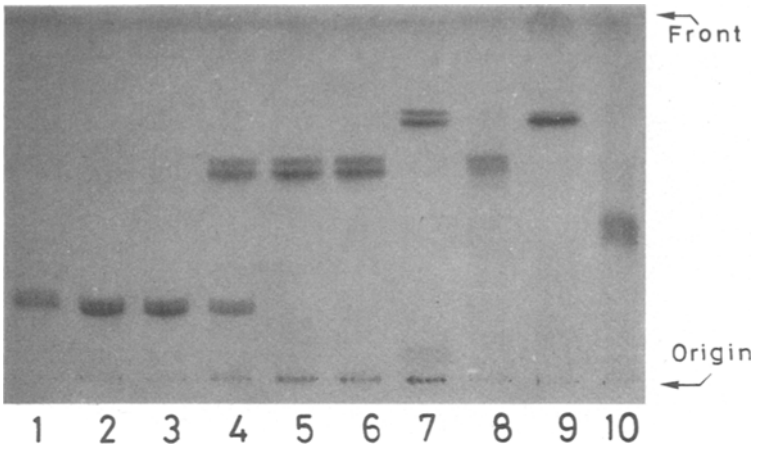

FIG. 2. Neuraminidase and β-galactosidase treatment of Ganglioside I. Ganglioside I (0.4 mg) was incubated with 20 mM acetate buffer (pH 5.0) only (lane 2), with 0.001 unit (lane 3), with 0.01 unit (lane 4) and with 0.1 unit (lane 5) of neuraminidase *(Cl. perfringens)* at 37 C for 3 hr. Desialylated ganglioside I (0.2 mg) isolated by preparative TLC was treated with the reaction buffer (0.2 M acetate, pH 4.0) only (lane 6) and with 0.01 unit of β-galactosidase *(C. lampas)* at 37 C for 15 hr (lane 7). 1, Ganglioside I; 8, LacCer; 9, GlcCer, 10, CTH. The plate was developed with solvent 3 ($CHCl_3/CH_3OH/H_2O$ [60:25:4, v/v/v]) and sprayed with α-naphthol-H_2SO_4.

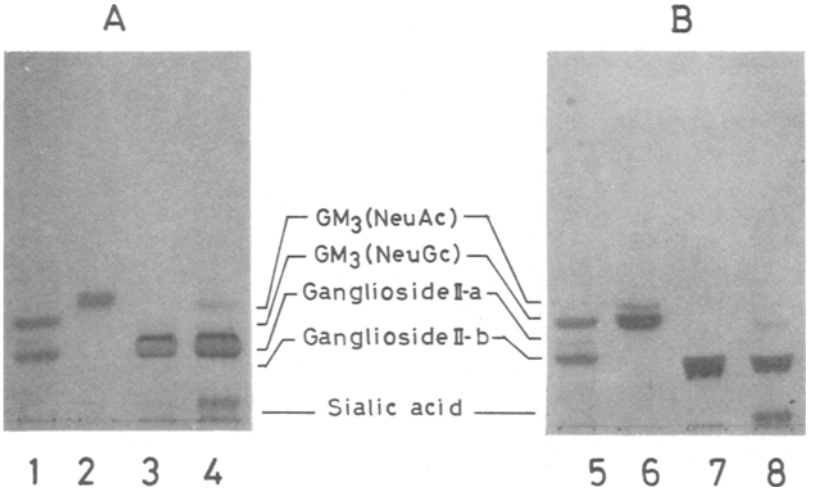

FIG. 3. Brief hydrolysis of Ganglioside II-a and II-b with neuraminidase *(Cl. perfringens)*. Ganglioside II-a (0.1 mg) was incubated with the reaction buffer only (lane 3) and with 0.05 unit of neuraminidase for 20 min at room temperature (lane 4). Ganglioside II-b (0.1 mg) was also treated with the reaction buffer only (lane 7), and 0.05 unit of neuraminidase (lane 8) for 20 min at room temperature. Lane 1 and lane 5, total gangliosides of porcine erythrocytes; lane 2, G_{M3}(NeuAc); lane 6, a mixture of G_{M3} (NeuAc type, 11%; NeuGc type, 89%). Plates A and B were developed with the solvent 2 ($CHCl_3/CH_3OH$/2.5 N NH_3 [60:35:8, v/v/v]) and sprayed with resorcinol-HCl reagent.

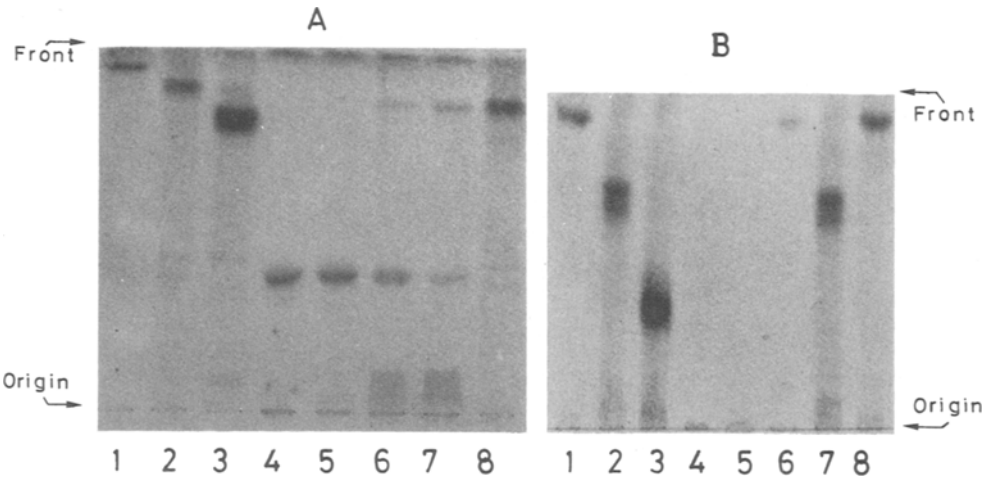

FIG. 4. Thin layer chromatography of the products obtained by hydrolysis of Ganglioside II with neuraminidase (A) and with neuraminidase followed by β-galactosidase (B). Ganglioside II (0.2 mg) was treated with the reaction buffer only (A, lane 4), with 0.001 unit (A, lane 5), with 0.01 unit (A, lane 6) and with 0.1 unit (A, lane 7) of *A. ureafaciens* neuraminidase at 37 C for 30 min. Products (0.1 mg) after incubation of Ganglioside II with 0.1 unit of the neuraminidase at 37 C for 30 min were incubated with the buffer only (B, lane 5) and with 0.1 unit of β-galactosidase *(C. lampas)* at 37 C for 14 hr (B, lane 6). Lane 1 in A, lane 1, 8 in B, GlcCer; lane 2, 8 in A, lane 2, 7 in B, LacCer; lane 3 in A and B, CTH; lane 4 in B, Ganglioside II. Plates were developed with solvent 1 ($CHCl_3/CH_3OH/H_2O$ containing 0.02% $CaCl_2 \cdot 2H_2O$ [60:40:9, v/v/v]) (A) and 3 ($CHCl_3/CH_3OH/H_2O$ [60:25:4, v/v/v]) (B), and sprayed with α-naphthol-H_2SO_4 reagent.

TABLE 3

Ganglioside Composition of Porcine Erythocyte Membranes

Gangliosides	μg/g dry stroma (% by weight)
G_{M3}(NeuAc) (NeuAcα2-3Galβ1-4Glcβ1-1ceramide)	16 ± 2 (2.6)
G_{M3}(NeuGc) (NeuGcα2-3Galβ1-4Glcβ1-1ceramide)	304 ± 42 (49.3)
G_{D3}(NeuAc) (NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ1-1ceramide)	30 ± 3 (4.9)
G_{D3}(NeuGc) (NeuGcα2-8NeuGcα2-3Galβ1-4Glcβ1-1ceramide)	240 ± 26 (38.9)
Others	27 ± 5 (4.3)

Values are means ± S.D. of the analyses of 4 different preparations. Gangliosides were isolated from the erythrocyte preparations (each 50 g) as described in the text, and the weight of each ganglioside was determined.

for influenza virus (24,25) and paramyxoviruses (26) have been demonstrated. Recently, G_{D3} gangliosides were reported as human melanoma associated antigen (27-30). Porcine erythrocytes may be a good source from which to prepare ganglioside G_{D3} in a sufficient quantity.

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Diversity of Sterol Composition in the Family Chenopodiaceae

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ABSTRACT

The predominant 4-desmethylsterols from the leaves of 13 species in eight genera of the family Chenopodiaceae are 24α-ethylsterols. In four species, *Chenopodium ambrosioides* L., *C. rubrum* L., *Salicornia europaea* L. and *S. bigelovii* Torr., the C-22(23) double bond is introduced into more than 70% of the 24α-ethylsterols producing spinasterol (24α-ethylcholesta-7,22E-dien-3β-ol) in the first two species and mixtures of spinasterol and stigmasterol (24α-ethylcholesta-5,22E-dien-3β-ol) in the latter species. The saturated side chain analogues predominate with more than 70% of the 24α-ethylsterols in eight species. *Salsola kali* L., *Suaeda linearis* (Ell.) Moq., *Kochia scoparia* (L.) Roth., and *Bassia hirsute* (L.) Aschers. synthesize sitosterol (24α-ethylcholest-5-en-3β-ol), and *Atriplex arenaria* Nutt., *C. album* L., *C. urbicum* L. and *C. leptophyllum* Nutt. possess mixtures of sitosterol and 22-dihydrospinasterol (24α-ethylcholest-7-en-3β-ol). Sitostanol (24α-ethyl-5α-cholestan-3β-ol) was isolated from *Suaeda linearis* as an 18% component of the total 4-desmethylsterol and in lesser amounts from four other species. In all species synthesizing 24-ethyl-Δ^5-sterols, a 24ξ-methylcholest-5-en-3β-ol was also present at 1.0-20% of the total 4-desmethylsterol. Avenasterol [24-ethylcholesta-7,24(28)Z-dien-3β-ol], isofucosterol [24-ethylcholesta-5,24(28)Z-dien-3β-ol], cholesterol (cholest-5-en-3β-ol) and 24ξ-methyl-5α-cholestan-3β-ol also were isolated from several species. Species in the family Chenopodiaceae and the type genus *Chenopodium* may be categorized into one of three groups based on sterol biosynthesis: the Δ^7-sterol producers; the Δ^5-sterol producers, and those producing mixtures of both Δ^7- and Δ^5-sterols in relatively fixed percentage compositions.
Lipids 20:594-601.

INTRODUCTION

Angiosperms biosynthesize a variety of 4-desmethylsterols with the majority of plant species reported to produce 24α-alkyl-Δ^5-sterols (1-4). However, species in the families Theaceae (tea) and Cucurbitaceae (cucumber) produce 24-alkyl-Δ^7-sterols (1,3) with the seed oils from *Trichosanthes kirilowii* (Cucurbitaceae) possessing a 25% and *Thea sinensis* (Theaceae) a 17% and the seeds of *Cucurbita maxima* (Cucurbitaceae) an 18% component of Δ^5-sterols (5-8). The families Solanaceae (potato), Brassicaceae (mustard), and Poaceae (grass) synthesize 24-alkyl-Δ^5-sterols with low levels of Δ^7-sterols present, presumably as biosynthetic intermediates (3). While the Asteraceae (aster) and Fabaceae (pea) are similar to the above four families (3), at least one species in each biosynthesizes exclusively Δ^7-sterols (9,10). Among the angiosperms in which more than one species within a genus was carefully examined, production of Δ^5- versus Δ^7-sterols in the photosynthetic tissue is consistent within a genus (11-13). In addition, the presence of Δ^5-sterols from the seed oils of various species within the Brassicaceae also are consistent with that reported for the leaves (14,15).

In the family Chenopodiaceae (goosefoot), the leaves of *Spinacia oleracea* (spinach) possess Δ^7-sterols, whereas the seed oil is reported to possess low levels, from <3% to 10% of Δ^5-sterols (6,10,16). The mature leaves of *Beta vulgaris* (beet) also in the Chenopodiaceae possess a repeatedly isolatable, relatively fixed mixture of low levels of Δ^5-sterols (30%) in the presence of high levels of Δ^7-sterols (17). Because both of these crop plants were selected and manipulated for their agricultural productivity, and because such selection may alter the production, content and composition of lipids in the plant (18), we examined the photosynthetic tissue of several non-crop-chenopods to determine the sterol biosynthetic capacity of the family. The sterols from the leaves of the 13 species examined provide data for a genetic diversity in sterol biosynthetic capacity within a family at the genus level. The angiosperm classification system of Cronquist (19), the subtribe arrangement of the Chenopodiaceae by Blackwell (20) and the nomenclature of Gray's Manual (21) are used in this paper.

MATERIALS AND METHODS

The plants were field-collected during the summer and fall of 1982 and 1983 as available, and all identifications were independently con-

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firmed by Dr. Albert List Jr., Department of Biological Sciences, Drexel University. Plants were cleaned of any necrotic tissue, and only the mature photosynthetic tissue (leaves and stems) was analyzed. The tissue was washed, finely chopped and extracted in a Soxhlet with acetone for 48 hr. The extracted material was saponified in 5% (w/v) KOH in 70% ethanol/water. The neutral lipids after saponification (NLAS) were extracted with ether and further fractionated by alumina chromatography using two void volumes each of hexane, hexane/benzene (1:1, v/v), benzene, ether, ether/methanol (1:1, v/v) and methanol. The sterols eluted in the ether fraction.

Sterols were further separated by preparative reverse-phase liquid chromatography (RPLC) on a Perkin-Elmer C_{18} High Efficiency Column and eluted with methanol/acetonitrile (1:9, 7.5 ml/min) at 35 C. Fractions were collected by eluting 150 ml from the column and then collecting 20 ml fractions for the next 600 ml. The sterols typically eluted in fractions 10-25. Fractions were evaporated under nitrogen and analyzed by GLC (XE-60) to produce an elution profile (Fig. 1). The 4-dimethylsterols were separated from the 4-desmethylsterols by TLC as previously described (22). Further separation of the 4-desmethylsterols was accomplished as previously described (17). The resultant purified sterols were then further characterized by mass and ^{1}H NMR spectroscopy.

GLC examination of sterols was performed with either 1% XE-60 on Chromosorb Q (100-120 mesh) at 230 C or 0.75% SE-30 on Chromosorb Q at 235 C in a Perkin-Elmer Sigma 3B with He 35 ml/min. RRT's are to cholesterol and agree with previously published values (17, 23). Analytical RPLC was performed on a Zorbax ODS column (30 cm x 3 mm) with isopropanol/acetonitrile (2:8, v/v) at 45 C, 1.5 ml/min on a Perkin-Elmer 3 liquid chromatograph with an LC-75 UV detector. Sample peaks were detected at 205 nm and scanned between 200 and 300 nm. The α_c (K′ sample/K′ cholesterol) were calculated as previously described (24). Electron impact mass spectroscopy was performed at 70 eV on a Finnigan model 4000 with a Series 6000 data system. ^{1}H NMR spectra were performed at 360 MHz at ambient temperature on a Bruker model WH360, in $CDCl_3$ with TMS as an internal standard.

Authentic standards were obtained and purified as previously reported (17) with additional standards from the following sources: isofucosterol, *Ulva lactuca*; fucosterol, *Laminaria* sp.; stigmastanol (sitostanol), Aldrich Chemical Co. (Milwaukee, Wisconsin); cholestanol and coprostanol, Supelco Inc. (Bellefonte, Pennsylvania); and campestanol, a gift from Dr. Karla S. Ritter.

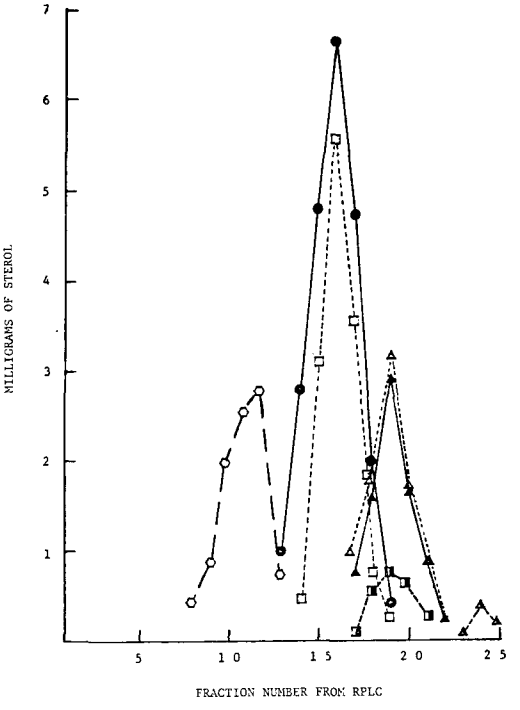

FIG. 1. Comparison of the RPLC profile of the sterols from *Salicornia europaea*. The RPLC fraction number (20 ml/fraction on the abscissa) is plotted versus the sterol content in mg of sterol per fraction (ordinate) as determined by GLC on XE-60. Cycloartenol (○), 24ξ-methylcholesterol and stigmasterol (□), sitosterol (△), spinasterol (●), 22-dihydrospinasterol (▲), 24ξ-methylcholestanol (◨), and sitostanol (▲).

RESULTS

The individually isolated sterols were characterized by their chromatographic properties on GLC and RPLC as well as their mass spectra and proton magnetic resonance spectra. The chromatographic characteristics are in agreement with previously published values (17,22,23) and with those of authentic standards concurrently tested. The configuration at C-24 for the epimeric 24α- and 24β-alkylsterols was determined through the use of proton nuclear magnetic resonance spectra and comparison with authentic standards run concurrently (7,8,15,29-31). The use of this technique provides consistently reproducible and distinctly different spectra for individual epimers as well as for mixtures of the C-24 epimers as previously reported (15,31).

Spinasterol (24α-ethyl-5α-cholesta-7,22E-dien-3β-ol) isolated from these chenopods had an RRT on SE-30 of 1.57 ± 0.01, on XE-60 of 1.52 ± 0.03 and an α_c of 1.10 ± 0.01. The mass spectra of spinasterol produced the following characteristic ions at m/e: 412 (M^+, 35%), 397 ($M-CH_3^+$, 27%), 379 ($M-CH_3-H_2O^+$, 19%), 369 ($M-C_3H_7^+$, 33%), 271 (M-side chain-$2H^+$, 98%), 255 (M-side chain-H_2O^+, 100%). The ^{1}H NMR spectra displayed the following chemical shifts in ppm from TMS for three protons at each carbon designated: C-18(s) 0.56, C-19(s) 0.80, C-21 (d, J=6Hz) 1.03, C-26,27 (d,d J=6Hz) 0.80 and 0.86, C-29 (t, J=7Hz) 0.81. The chemical shifts for protons of authentic chondrillasterol (24β-epimer) are sufficiently different, 0.83 and 0.85 for C-26 and C-27 to confirm the α-configuration (7,17).

The 22-dihydrospinasterol or schottenol (24α-ethyl-5α-cholest-7-en-3β-ol) isolated from these chenopods had an RRT on SE-30 of 1.81 ± 0.02, on XE-60 of 1.71 ± 0.06 and an α_c of 1.26 ± 0.02. The mass spectrum of 22-dihydrospinasterol produced the following characteristic ions at m/e: 414 (M^+, 99%), 399 ($M-CH_3^+$, 74%), 381 ($M-CH_3-H_2O^+$, 16%), 273 (M-side chain$^+$, 78%), 255 (M-side chain-H_2O^+, 100%). The ^{1}H NMR spectra displayed the following chemical shifts in ppm from TMS for three protons at each carbon designated: C-18(s) 0.54, C-19(s) 0.80, C-21 (d, J=6Hz) 0.94, C-26,27 (d,d, J=6Hz) 0.82 and 0.84, C-29 (t, J=7Hz) 0.85. The chemical shifts for protons of authentic 22-dihydrochondrillasterol (24β-epimer) are sufficiently different, 0.55 for C-18 and 0.86 for C-29, to confirm the α-configuration (7,17) and produce non-superimposable spectra.

Avenasterol [24-ethyl-5α-cholesta-7,24(28)Z-dien-3β-ol] isolated from these chenopods had an RRT on SE-30 of 1.87 ± 0.01, on XE-60 of 1.88 ± 0.02 and an α_c of 0.93 ± 0.01. The mass spectrum of avenasterol produced the following characteristic ions at m/e: 412 (M^+, 26%), 397 ($M-CH_3^+$, 24%), 379 ($M-CH_3-H_2O^+$, 25%), 314 ($M-C_7H_{14}^+$, 92%), 299 ($M-C_7H_{14}-CH_3^+$, 49%), 271 (M-side chain-$2H^+$, 100%), 255 (M-side chain-H_2O^+, 82%). The ion at m/e 314 with its high relative abundance is characteristic of a 24(28) double bond (28). The ^{1}H NMR spectra displayed the following chemical shifts in ppm from TMS for three protons at each carbon designated: C-18(s) 0.54, C-19(s) 0.80, C-21 (d, J=6Hz) 0.95, C-26,27 (d,d J=6Hz) 0.98 and 0.98, C-29 (d, J=6Hz) 1.58. The H-25 multiplet was observed at 2.83 ppm, which is in agreement with that reported for the Z isomer of the 24(28)-bond (7,25,28).

Sitosterol (24α-ethylcholest-5-en-3β-ol) isolated from these chenopods had an RRT on SE-30 of 1.62 ± 0.01, on XE-60 of 1.55 ± 0.02 and an α_c of 1.23 ± 0.01. The mass spectra of sitosterol produced the following characteristic ions at m/e: 414 (M^+, 92%), 399 ($M-CH_3^+$, 61%), 396 ($M-H_2O^+$, 43%), 381 ($M-CH_3-H_2O^+$, 16%), 303 ($M-C_7H_9-H_2O^+$, 61%), 273 (M-side chain$^+$, 61%), 255 (M-side chain-H_2O^+, 100%). The ^{1}H NMR spectra displayed the following chemical shifts in ppm from TMS for three protons at each carbon designated: C-18(s) 0.68, C-19(s) 1.01, C-21 (d, J=6Hz) 0.92, C-26,27 (d, d, J=6Hz) 0.81 and 0.83, C-29 (t, J=7Hz) 0.85. These values are in agreement with authentic sitosterol and previously published values (7,17, 22,25-27).

Isofucosterol [24-ethylcholesta-5,24(28)Z-dien-3β-ol] isolated from these chenopods had an RRT on SE-30 of 1.61 ± 0.01, on XE-60 of 1.54 ± 0.02 and an α_c of 0.93 ± 0.01. The mass spectrum for isofucosterol produced ions at m/e: 412 (M^+, 10%), 397 ($M-CH_3^+$, 4%), 394 ($M-H_2O^+$, 4%), 379 ($M-CH_3-H_2O^+$, 3%), 314 ($M-C_7H_{14}^+$, 100%), 299 ($M-C_7H_{14}-CH_3^+$, 27%), 281 ($M-C_7H_{14}-CH_3-H_2O^+$, 28%), 271 (M-side chain-$2H^+$, 36%), 255 (M-side chain-H_2O^+, 21%). The ion at m/e 314 with its relative abundance as a base peak is characteristic of the 24(28) double bond (28). The ^{1}H NMR spectra displayed the following chemical shifts from TMS for three protons at each carbon designated: C-18(s) 0.69, C-19(s) 1.01, C-21 (d, J=6Hz) 0.95, C-26,27 (d, d, J=6Hz) 0.98 and 0.98, C-29 (d, J=6Hz) 1.58. The superimposed doublets for the H on C-26, C-27 in the ^{1}H NMR spectra are consistent with the *cis* or Z isomer for the 24(28)-double bond (26,27). The *trans* or E isomer as present in fucosterol has distinctly different signals for these hydrogens. The ^{1}H NMR spectrum of authentic fucosterol displayed the following chemical shifts in ppm from TMS for three protons at each carbon designated: C-18(s) 0.69, C-19(s) 1.02, C-21 (d, J=6Hz) 0.98, C-26,27 (d, d, J=6Hz) 1.20 and 1.22, C-29 (d, J=6Hz) 1.58.

Stigmasterol (24α-ethylcholesta-5,22 E-dien-3β-ol) isolated from these chenopods had an RRT on SE-30 of 1.42 ± 0.02, on XE-60 of 1.32 ± 0.02 and an α_c of 1.07 ± 0.01. The mass spectrum for stigmasterol produced the following characteristic ions at m/e: 412 (M^+, 57%), 397 ($M-CH_3^+$, 17%), 394 ($M-H_2O^+$, 13%), 379 ($M-CH_3-H_2O^+$, 17%), 369 ($M-C_3H_7^+$, 22%), 299 ($M-C_7H_{11}-H_2O^+$, 53%), 271 (M-side chain-$2H^+$, 75%), 255 (M-side chain-H_2O^+, 100%). The ^{1}H NMR spectra displayed the following chemical shifts in ppm from TMS for three protons at each carbon designated: C-18(s) 0.70, C-19(s) 1.01, C-21 (d, J=6Hz) 1.03, C-26,27 (d,d, J=6Hz) 0.80 and 0.85, C-29 (t, J=7Hz) 0.81. The chemical shift of 1.03 for the protons on C-21

is indicative of a 22(23) double bond (29) and similar chemical shifts for the protons on C-26 and C-27 to those found in spinasterol and not those of chondrillasterol are indicative of a 24α-ethyl group.

The 24ξ-methylcholest-5-en-3β-ol isolated from these chenopods had an RRT on SE-30 of 1.30 ± 0.01, on XE-60 of 1.30 ± 0.02 and an α_c of 1.10 ± 0.01. The mass spectrum of this component produced ions at m/e: 400 (M^+, 65%), 385 ($M-CH_3^+$, 18%), 382 ($M-H_2O^+$, 26%), 367 ($M-CH_3-H_2O^+$, 18%), 315 ($M-C_6H_{13}^+$, 26%), 289 ($M-C_7H_9-H_2O^+$, 49%), 273 (M-side chain$^+$, 46%), 255 (M-side chain-H_2O^+, 100%). The ^{1}H NMR of the 24-methylcholesterol isolated from all these chenopods produced broad signals for H on C-18 and a multiple of doublets in the 0.75-0.85 ppm region of the spectrum. These signals are indicative of epimeric mixtures (30, 31) of 24α-methylcholesterol (campesterol) and 24β-methylcholesterol(22-dihydrobrassicasterol) the 360 MHz ^{1}H NMR spectra of which are presented in Ref. 22. Since plants which synthesize 24-methylcholesterol are known to produce epimeric mixtures (30), further analysis of these fractions was not performed.

Cholesterol was identified in two species of chenopods and had an RRT on SE-30 of 1.00 ± 0.01, on XE-60 of 1.00 ± 0.02 and an α_c of 1.00 ± 0.01. The mass spectra and ^{1}H NMR spectra agreed with those obtained from a standard of cholesterol. A 24-methylcholestadienol also was identified in two species of chenopods by its chromatographic and mass spectral properties. This component had an RRT on SE-30 of 1.16 ± 0.04, on XE-60 of 1.12 ± 0.03 and an α_c of 0.90 ± 0.02. Due to the limited quantity of this compound, ^{1}H NMR spectroscopy was not performed.

Sitostanol (24α-ethyl-5α-cholestan-3β-ol) was identified in five species of chenopods. This compound, which has GLC properties similar to those of the Δ^5-analogue sitosterol, was separated by the preparative RPLC column (Fig. 1) and subsequently was detected by GLC of the RPLC fractions. The α_c of sitosterol is 1.23 ± 0.01, whereas the estimated α_c of sitostanol is 1.41-1.44. The value is estimated by extrapolation from the GLC reinjection of the preparative RPLC fractions, because the stanols do not have an absorbance at 205 nm and no peak is observed from the RPLC UV detection system. Analysis by mass spectroscopy of what appeared originally to be a tail on the sitosterol peak provided the spectrum of sitostanol with the following ions at m/e: 416 (M^+, 31%), 401 ($M-CH_3^+$, 10%), 398 ($M-H_2O^+$, 2%), 383 ($M-CH_3-H_2O^+$, 4%), 344 ($M-C_4H_6-H_2O^+$, 1%), 275 (M-side chain$^+$, 2%), 257 (M-side chain-H_2O^+, 7%), 248 ($M-168^+$, 14%), 233 ($M-183^+$, 78%), 215 ($M-201^+$, 100%). This mass spectrum is identical with that of an authentic standard of sitostanol and previously published values (32).

The ^{1}H NMR spectroscopy of cholestanol (5α-cholestan-3β-ol), coprostanol (5β-cholestan-3β-ol) and sitostanol (24α-ethyl-5α-cholestan-3β-ol) standards was performed to ascertain the effect of the planarity (5α-H) of the A/B ring juncture or lack thereof (5β-H) on the ^{1}H NMR signals at 360 MHz. The introduction of the 5β-hydrogen exhibits the greatest effect on the signals for the protons on C-19. The chemical shift for the H on C-19 of cholestanol (5α-H) is 0.80, while for coprostanol (5β-H) it is 0.96. The sitostanol standard also exhibited a chemical shift for the C-19 hydrogens at 0.80 ppm. The sitostanol isolated from the chenopods displayed the following chemical shifts in ppm from TMS for three protons at each carbon designated: C-18(s) 0.65, C-19(s) 0.80, C-21 (d, J=6Hz) 0.91, C-26,27 (d,d, J=6Hz) 0.81 and 0.83, C-29 (t, J=7Hz) 0.84. This ^{1}H NMR spectrum was identical to that obtained for the authentic sitostanol standard.

A 24ξ-methyl-5α-cholestan-3β-ol also was isolated from five chenopods and was detected by GLC of the RPLC fractions obtained from the preparative RPLC column (Fig. 1). The estimated α_c of this component is 1.23-1.26, whereas the α_c for campesterol, the 24α-methyl-Δ^5-sterol, is 1.10. The preparative RPLC separated the 24-methyl- and 24-ethylstanols from their Δ^5-analogues (Fig. 1). The ^{1}H NMR of the 24ξ-methyl-5α-cholestan-3β-ol produced a signal for the C-19 protons at 0.80 ppm indicative of a 5α-H at the A/B ring juncture. The signals from the protons at C-26, C-27 and C-28 appear as a multiple of doublets from 0.77-0.85 ppm similar to those observed in the epimeric mixtures in the 24-methyl-Δ^5-sterols. These 24-methyl-5α-cholestanols are therefore believed to be a C-24 epimeric mix. The mass spectrum of this 24ξ-methyl-5α-cholestan-3β-ol produced ions at m/e: 402 (M^+, 35%), 387 ($M-CH_3^+$, 17%), 369 ($M-CH_3-H_2O^+$, 6%), 257 (M-side chain-H_2O^+, 7%), 248 ($M-154^+$, 11%), 233 ($M-169^+$, 82%) and 215 ($M-187^+$, 100%). This is in agreement with previously reported values (32).

Cycloartenol (9β,19-cyclo-4,4,14α-trimethylcholest-24-en-3β-ol) was isolated and identified by its chromatographic properties from five species of chenopods including *Salicornia europaea* (Fig. 1). Cycloartenol had an RRT on SE-30 of 1.81 ± 0.04, on XE-60 of 1.71 ± 0.08 and an α_c of 0.90 ± 0.04. The mass spectrum produced characteristic ions at m/e: 426 (M^+, 27%), 411 ($M-CH_3^+$, 53%), 408 ($M-H_2O^+$, 16%), 393 ($M-CH_3-H_2O^+$, 29%), 365 ($M-C_3H_7-H_2O^+$, 7%),

339 ($M-C_5H_9-H_2O^+$, 5%), 314 (M-side chain$^+$, 14%), 299 (M-side chain-CH_3^+, 6%), 286 ($M-C_9H_{14}-H_2O^+$, 35%), 271 ($M-C_9H_{14}-CH_3-H_2O^+$, 100%) and 259 ($M-C_{12}H_{23}^+$, 10%). The ^{1}H NMR produced the following chemical shifts in ppm from TMS for protons at the carbons designated: 3H at C-30 (C-4α, s) 0.96, 3H at C-31 (C-4β, s) 0.96, 3H at C-32 (C-14α, s) 0.89, 3H at C-18(s) 0.81, 2H at C-19 (d,d J=6Hz) 0.33 and 0.55, 3H at C-21 (d, J=6Hz) 0.88, 3H at C-26(s) 1.60, 3H at C-27(s) 1.68. The upfield doublets for the two protons on C-19 in the cyclopropane ring are characteristic of cycloartenol (46). Chemical shifts of 1.60 and 1.68 for protons on C-26 and C-27, respectively, confirm the presence of the 24(25) double bond. The mass and NMR spectra were similar to those previously reported for cycloartenol (10,45,47).

Lanosterol (4,4,14α-trimethylcholesta-8(9), 24-dien-3β-ol) was isolated and identified by its chromatographic characteristics from *Salicornia bigelovii*. Lanosterol had an RRT on SE-30 of 1.60 ± 0.02, on XE-60 of 1.48 ± 0.04 and an α_c of 0.81 ± 0.01. The mass spectrum produced characteristic ions at m/e: 426 (M^+, 49%), 411 ($M-CH_3^+$, 100%), 393 ($M-CH_3-H_2O^+$, 39%), 273 ($M-C_{10}H_{15}-H_2O^+$, 15%) and 259 ($M-C_{12}H_{23}^+$, 19%). The ^{1}H NMR spectra displayed the following chemical shifts in ppm from TMS for three protons at the carbons designated: C-30 (C-4α, s) 0.98, C-31 (C-4β, s) 0.81, C-32 (C-14α, s) 0.88, C-18(s) 0.69, C-19(s) 1.00, C-21 (d, J= 6Hz) 0.91, C-26(s) 1.61 and C-27(s) 1.67. The mass and NMR spectra were similar to those previously reported for lanosterol (33).

DISCUSSION

The photosynthetic tissues of plants in the family Chenopodiaceae possess a diversity of sterol biosynthetic capacity previously unknown in any one family of angiosperms. The dominant 4-desmethylsterols in all thirteen chenopod species are the 24α-ethylsterols. Within the genus *Chenopodium* in the subtribe Chenopodioideae, *C. ambrosioides* and *C. rubrum* produce exclusively Δ^7-sterols (Table 1) and thus are similar in sterol composition to species in the family Cucurbitaceae (1). However, the species *Chenopodium album*, *C. urbicum* and *C. leptophyllum* (Table 1) produce mixtures of Δ^5-sterols and Δ^7-sterols in approximately equal proportions. Duplicate samples of these plants collected in different years and/or grown in the greenhouse provided similar sterol profiles and composition within the variation reported for samples of *Beta vulgaris* (17). Similarly, *Salicornia europaea* and *S. bigelovii* (Table 1, Fig. 1) also produce mixtures of both Δ^5- and Δ^7-sterols in approximately equal proportions. However, these *Salicornia* species differ from the three Δ^5- and Δ^7-sterol-producing *Chenopodium* species in their degree of sterol side chain unsaturation. The *Salicornia* species possess high levels of side chain unsaturation, i.e. >60% of the total 4-desmethylsterol has C-22 or C-24(28) double bonds, whereas the three *Chenopodium* species have more highly saturated side chains. These variations do not appear to be attributed to the age of the tissues, as both juvenile and mature leaves and stems possess similar 4-desmethylsterol compositions. Juvenile photosynthetic tissue of *C. album* was found to contain, based on TLC, GLC and mass spectral data, various 4,4-dimethyl- and 4-monomethyl-sterols which included cycloartenol 0.8%, obtusifoliol 1.0% and 4α,14α-dimethylcholesta-8(9),24(25)-dien-3β-ol 7.3%, that were not isolated from mature tissue.

In four of the five *Chenopodium* and *Salicornia* species which produce mixtures of Δ^5- and Δ^7-sterols, stanols either 24ξ-methyl-5α-cholestanol and/or 24α-ethyl-5α-cholestanol were present (Table 1). These isolated stanols all possess a *trans* A/B ring juncture as observed in the ^{1}H NMR spectra and probably are of plant biosynthetic origin. Since no stanols are detectable in chenopod species producing only Δ^7-sterols, and the 5α-stanols are present in Δ^5-sterol producing chenopods, these 5α-stanols presumably are synthesized from a Δ^5-sterol as demonstrated in other organisms (34-36).

The species *Atriplex arenaria, Bassia hirsute* and *Kochia scoparia* in the subtribe Chenopodioideae and *Salsola kali* and *Suaeda linearis* in the subtribe Salsoloideae (20) all produce 24α-ethyl-Δ^5-sterols as their dominant sterols, i.e. >70% of the total 4-desmethylsterols (Table 1). Low levels of the Δ^7-sterols, avenasterol (3-5%) in *A. arenaria, B. hirsute* and *S. kali* and the 16% component of 22-dihydrospinasterol in *A. arenaria*, are well within the range, usually <20% of the total 4-desmethylsterols, assumed to be biosynthetic intermediates that occur in "main line," i.e. Δ^5-sterol-producing plants (1). Sitosterol is, as a percentage of the total 4-desmethylsterol, the dominant sterol in these five species but comprises from 39 to 87% of this total (Table 1). High levels of isofucosterol (19%) in *B. hirsute* and isofucosterol (16%) and sitostanol (19%) in *S. linearis* greatly depress the contribution of sitosterol as a percentage of the total in these species. In these five species, with the exception of *B. hirsute*, the 24-ethylsterols with a saturated side chain predominate by 3- to 8-fold over the 24-ethylsterols with unsaturated side chains, i.e. Δ^{22} and $\Delta^{24(28)}$ sterols. The ratio of saturated to unsaturated side chain

TABLE 1

Percent Composition of the 4-Desmethylsterols in the Chenopodiaceae

	Cholesterol	24-Methyl-cholesta-dienol	Isofucosterol	24ξ-Methyl cholesterol	Stigmasterol	Sitosterol	Avenasterol	Spinasterol	22-Dihydro-spinasterol	24ξ-Methyl-cholestanol	Sitostanol
Chenopodioideae											
Chenopodium ambrosioides							22.5	67.3	10.2		
Chenopodium rubrum							23.0	54.4	22.6		
Chenopodium album	3.5	5.0		2.4	10.0	23.8		15.6	37.7	1.1	0.9
Chenopodium urbicum		1.5		2.2	7.1	41.1	1.8	7.1	38.3	0.9	
Chenopodium leptophyllum				1.0	10.0	45.6	1.5		41.9		
Salicornia europaea				4.4	24.7	15.1		37.7	12.9	4.3	1.0
Salicornia bigelovii			18.2	1.8	29.4	1.1		31.5	9.9	3.6	4.5
Atriplex arenaria				3.4	24.7	52.6	3.0		16.3		
Beta vulgaris				6.5	7.1	13.0	1.4	47.0	25.0		
Bassia hirsute	4.5		19.2	10.7	15.1	45.5	5.0				
Kochia scoparia				2.0	10.5	87.5					
Salsoloideae											
Salsola kali				20.7	3.2	67.8	2.7				5.6
Suaeda linearis			15.8	6.5	13.5	39.8				5.7	18.7

in the 24-ethylsterols in *B. hirsute* is approximately one. The presence of both isofucosterol (19%) and avenasterol (5%) in the leaf tissue of *B. hirsute* is atypical because in the other 12 species only one or the other of these biosynthetic intermediates is present (Table 1). Reports of the co-occurrence of these sterols are confined to seed or seed products and not to leaf tissue (6,3 and references therein).

The relatively high levels of sitostanol in *Suaeda linearis* and *Salsola kali* are the presumed product of further metabolism of a Δ^5-sterol and may be characteristic of plants in this subtribe. Whatever the metabolic sequence, the process appears to be under a different kinetic control in the case of *S. kali* and *S. linearis* when compared with the two *Chenopodium* and the two *Salicornia* species which produce stanols. The latter four species produce, as well as stanols, mixtures of Δ^7- and Δ^5-sterols in approximately equal proportions, whereas the two former species synthesize Δ^5-sterols. The apparent regulation of the 1:1 mixtures of Δ^7- and Δ^5-sterols in *Chenopodium* and *Salicornia*, which were sampled and analyzed several times, may be the result of different end product requirements in these species. Several functions for sterols are proposed in *Saccharomyces cerevisiae*, of which one function has a high stereospecificity and requires ergosterol while the bulk functions can be satisfied by other sterols of varying structure (37-43). Presumably similar functions and thus requirements for sterols with some altered structure or stereospecificity exist in plant cells (44). The diversity and ratios of sterols in these Chenopodiaceae species may reflect differing functional requirements in these plants or may be indicative of an evolutionary progression in the regulation of this pathway. Some species in the Chenopodiaceae, including *Chenopodium rubrum*, *Bassia hirsute*, *Atriplex arenaria*, both *Salicornias*, *Suaeda linearis* and *Salsola kali*, are halotolerant, and many species are highly adaptable weeds found in waste places. A plant which is capable of tolerating harsh and widely varying conditions may adapt its membrane functions more readily if sterols of differing structure are available. If, however, this diversity reflects the evolution of regulation within the sterol pathway, the species in the genus *Chenopodium* could serve as a useful tool for investigating these control mechanisms. From a regulatory perspective it is of interest that several species maintained relatively high levels of 4,4,14α-trimethylsterol precursors in their mature tissue. Cycloartenol was isolated from the mature leaves of the following five species and is reported as a percentage of the total sterol isolated: *Salicornia europaea*, 14%; *Bassia hirsute*, 4%; *Kochia scoparia*, 13%; *Beta vulgaris*, 2.8%; and *Suaeda linearis*, 30%. Lanosterol was isolated from *Salicornia bigelovii*, 8%. The lanosterol presumably is the cleavage product of the 9,19-cyclopropyl ring system of cycloartenol. Extensive examination of the mature plant, however, did not reveal any cycloartenol.

It is of interest that both species of *Salicornia* maintain these biosynthetic precursors, yet none were found in the genus *Chenopodium* in spite of a similarity in 4-desmethylsterol content among the species in these two genera. These sterol intermediates presumably are under different regulatory controls in the two genera. The unusual 7:3 ratio of Δ^7- to Δ^5-sterol observed in *Beta vulgaris* leaves (17) and in the seed oils of two species of *Gleditsia* (Caesalpiniceae) (6) may also reflect intermediate regulatory or kinetic steps. Further insight into the genetic and regulatory mechanisms of angiosperm phytosterol biosynthesis may be gained by a careful examination of this unusually diverse plant family and, in particular, the genus *Chenopodium*.

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Role of Prostanoids and Lipid Peroxides as Mediators of the 12-O-Tetradecanoylphorbol-13-Acetate Effect on Cell Growth[1]

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ABSTRACT

Confluent cultures of guinea pig smooth muscle cells (SMC) or human fibroblasts (HNF) were treated with 12-tetradecanoylphorbol-13-acetate (TPA). Prostanoid levels were measured by the radioimmunoassay of 6-keto-$PGF_{1\alpha}$ and PGE_2, and lipid peroxides were measured by the thiobarbituric acid test for malondialdehyde (MDA). Cells were seeded at low densities, and growth was calculated both from the cell count (Coulter Counter) and the colony number (image analysis). When confluent SMC and HNF were incubated in media alone, 6-keto-$PGF_{1\alpha}$ levels were a function of the TPA concentration, increasing to a maximum at 10^{-8} M TPA and then decreasing at higher TPC concentrations. When confluent SMC and HNF were incubated in media containing exogenous arachidonic acid, 6-keto-$PGF_{1\alpha}$ levels again increased to a maximum at 10^{-8} M TPA but decreased at higher TPA concentrations only with SMC. The increase in 6-keto-$PGF_{1\alpha}$ levels was much greater in HNF (1310%) than SMC (680%). SMC synthesized similar amounts of 6-keto-$PGF_{1\alpha}$ and PGE_2, and the stimulatory effect of TPA was similar with 6-keto-$PGF_{1\alpha}$ and PGE_2. Indomethacin (IM) blocked prostanoid synthesis at all TPA concentrations. TPA did not have a significant effect on MDA levels in either cell line. The lipid antioxidants α-tocopherol and α-tocopherylquinone blocked lipid peroxidation without affecting the stimulation of prostanoid synthesis with TPA. Cell number decreased to a minimum at 10^{-8} M TPA in both cell lines. The decrease in cell number was much greater in HNF (72%) than SMC (30%). SMC colony number decreased at 10^{-8} TPA and then increased at 10^{-6} M TPA. IM did not block the TPA effect on cell number in either cell line. The lipid antioxidant butylated hydroxytoluene (BHT) did not block the TPA effect on SMC cell number. The IM and the BHT data show that the TPA effect on cell growth is not mediated by prostanoid or lipid peroxide products of arachidonic acid metabolism. However, the increase in prostanoid synthesis parallels the decrease in cell number, and the effects are maximal at the same TPA concentration. These correlations suggest a common cellular process affecting both prostanoid synthesis and cell growth that is initiated or enhanced by TPA.
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INTRODUCTION

A number of studies from our laboratory have sought to identify the different effects that essential fatty acids and other polyunsaturated fatty acids have on cell proliferation in smooth muscle cells (SMC) and fibroblasts (HNF) (1-15). These effects, which depend on fatty acid structure and concentration (1-3,8, 12,13), are closely related to the synthesis of prostanoids and lipid peroxides in these cell lines. Small increments in prostanoid levels enhance cell growth, and large increments in prostanoid levels diminish cell growth (1,3,6-9, 12-14). Lipid peroxides are associated only with a concentration-dependent decrease in cell growth (3-7,9,12-15).

The tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) stimulates cellular oxidation reactions that generate both superoxide and hydrogen peroxide (13,16-22). TPA also enhances fatty acid release from cellular phospholipids (23-26) and stimulates both cyclooxygenase and lipoxygenase reactions (23-30). TPA functions not only as a tumor promoter but also as an agent that affects cellular differentiation and proliferation (13,16,24,25,31-35). Several investigators have suggested that TPA mediates cellular processes such as growth and differentiation through the cyclooxygenase and lipoxygenase products of arachidonic acid [20:4 (n-6)] metabolism (13,16,24,25,28-30,36-41). However, cells exhibit pleiotropic responses to TPA (16,36), and it has been difficult to distinguish between the possibility that the TPA effect on growth is mediated by 20:4(n-6) metabolism and the possibility that the TPA effects on growth and 20:4(n-6) metabolism are merely concurrent cellular events. These questions are addressed in the present study by examining the effect of TPA concentration on both cell growth and 20:4(n-6) metabolism.

[1] This publication is Part VIII in the series, "Fatty Acid Metabolism and Cell Proliferation."

MATERIALS AND METHODS

Materials

TPA was obtained from Dr. P. Borchert (University of Minnesota, Minneapolis, Minnesota). 20:4(n-6) was purchased from NuChek Prep (Elysian, Minnesota) and purified as previously described (1). Indomethacin (IM) was purchased from Sigma Chemical Co. (St. Louis, Missouri). Antisera for the radioimmunoassay (RIA) of 6-keto-$PGF_{1\alpha}$ and PGE_2 were supplied by Dr. L. Levine (Brandeis University, Waltham, Massachusetts).

Tissue Culture

Primary cultures of SMC were obtained from the dissected medial layer of guinea pig aorta obtained from prepubertal males, and primary cultures of HNF were obtained from neonatal foreskin. The experimental details of our tissue culture procedures are provided elsewhere (2,4, 5,42,43). The medium for growing cells to confluency (Growth Medium), the medium in TPA experiments with confluent cultures (Experimental Medium) and the medium in cell proliferation experiments (Cloning Medium) have been described (14). TPA was dissolved in acetone, and other agents were dissolved in 95% ethanol prior to dilution with Experimental or Cloning Media. Control cultures were treated with media containing the same amount of ethanol (1:500) or acetone (1:500).

Prostanoids and Lipid Peroxides

SMC or HNF were seeded at 2.5×10^5 cells per flask and grown to confluency in Corning T-25 flasks containing 4 ml of Experimental Medium. Cells were treated with fresh media containing the specific agent(s) and incubated for 4 hr or 24 hr.

Prostanoids were measured by a standard radioimmunoassay (RIA) procedure (44). Culture media was used directly because preliminary experiments showed that extraction did not affect the results. The RIA data were confirmed by the extraction and separation of prostanoids by high performance liquid chromatography (14). A standard curve was generated for each RIA experiment. Media from each flask were analyzed at several dilutions, and only data from the linear portion of the standard curve were used in calculations. The reproducibility of the RIA procedure was estimated by the coefficient of variation (S.D./mean in %; C.V.). C.V. values were 4.9, 5.7 and 7.5 for six replicate measurements of media in each of three flasks grown at the same time from the same primary culture. The C.V. was 6.0 for the mean of the mean values from each flask. The cross-reactivity of the 6-keto-$PGF_{1\alpha}$ antibody with related compounds was as follows: PGE_2, 0.15%; PGD_2, 0.02%; $PGF_{2\alpha}$, 0.10%; 20:4(n-6), 0.005%. Cross-reactivity of the PGE_2 antibody with related compounds was as follows: 6-keto-$PGF_{1\alpha}$, 0.4%; PGF_1, 0.76%; PGF_2, 0.31%; PGD_2, 0.051%; 20:4(n-6), 0.00045%. Data for the immunoreactive metabolite are expressed as nmol/culture.

Lipid peroxides were assayed by the thiobarbituric acid (TBA) test as previously described (5,14). The absorbance at 532 nm was measured and is reported as nmoles of malondialdehyde (MDA)/culture.

Cell Proliferation

SMC or HNF were passaged serially at a cell density of around 40 cell/cm^2. Cells were allowed to attach for one day and then treated with fresh media containing the specified agent(s). After an 8- to 10-day incubation (cells were re-fed on day 5) cultures were washed with Hank's balanced salt solution at pH 7.2, incubated at 37 C for 5 min with 2 ml of 0.1% trypsin, detached cells collected by centrifugation, and the cell count determined with a Coulter Counter (Coulter Electronics, Hialeah, Florida).

The number of colonies was obtained by image analysis using an Optomax Visual Analysis System (Optomax, Wallis, New Hampshire) equipped with an automatic chord sizing module. This technique (6,9,12) generated the complement of a cumulative distribution function which showed a decreasing number of colonies with increasing colony size. Image analysis was not used to estimate cell number, because preliminary experiments confirmed other studies which showed that TPA decreased cell size (16).

Statistics

All data are reported as mean ± SEM. The significance of differences in a treatment series was determined by a one-way analysis of variance (F-statistic). Individual treatments were compared with the control by the Tukey-HSD test.

RESULTS

Prostanoids

Prostanoid synthesis in SMC cultures, measured as the 6-keto-$PGF_{1\alpha}$ level in the media, increased during a 24-hr incubation period. Data at 4 hr and 24 hr are reported in Table 1. The prostanoid level in SMC cultures was a function of the TPA concentration in the culture, increasing to a maximum at 10^{-8}M TPA and then

TABLE 1

Effect of TPA on Prostanoid Synthesis (6-keto-$PGF_{1\alpha}$) and Lipid Peroxidation (MDA) in Confluent Cultures of SMC

TPA (M)	Incubation time 4 hr	24 hr
	6-keto-$PGF_{1\alpha}$	
0	0.14 ± 0.02(8)[a]	0.20 ± 0.02(7)
10^{-10}	0.16 ± 0.03(4)	0.30 ± 0.03(4)
10^{-9}	0.23 ± 0.02(4)	0.53 ± 0.03(4)
10^{-8}	0.31 ± 0.01(8)[b]	1.36 ± 0.11(8)[b]
10^{-7}	0.24 ± 0.02(8)[c]	0.92 ± 0.12(8)[b]
10^{-6}	0.19 ± 0.02(8)	0.37 ± 0.01(8)
F ratio	10.67	31.15
	MDA	
0	0.42 ± 0.07(8)	0.89 ± 0.09(10)
10^{-10}	0.58 ± 0.09(4)	0.72 ± 0.18(4)
10^{-9}	0.54 ± 0.05(4)	0.87 ± 0.27(4)
10^{-8}	0.50 ± 0.02(10)	1.32 ± 0.09(10)
10^{-7}	0.54 ± 0.08(7)	1.17 ± 0.11(8)
10^{-6}	0.45 ± 0.08(7)	0.94 ± 0.14(8)
F ratio	0.77	2.95

[a]Concentration in nmoles/culture; mean ± SEM; number of experiments in parentheses.

[b]Significantly higher (Tukey-HSD test) than both media alone and highest TPA concentration.

[c]Significantly higher (Tukey-HSD test) than media alone.

TABLE 2

Effect of TPA on Prostanoid Synthesis (6-keto-$PGF_{1\alpha}$) and Lipid Peroxidation (MDA) in Confluent Cultures of HNF

TPA(M)	6-keto-$PGF_{1\alpha}$	MDA
0	0.08 ± 0.002(6)[a]	0.64 ± 0.09(5)
10^{-10}	0.11 ± 0.003(6)	0.55 ± 0.07(6)
10^{-9}	0.30 ± 0.007(6)[b]	0.57 ± 0.06(6)
10^{-8}	1.05 ± 0.04 (6)[c]	0.93 ± 0.15(6)
10^{-7}	0.79 ± 0.04 (6)[b]	0.77 ± 0.13(6)
10^{-6}	0.56 ± 0.03 (6)[b]	0.70 ± 0.07(6)
10^{-5}	0.55 ± 0.04 (6)[b]	0.50 ± 0.07(6)
F ratio	154.3	2.39

[a]Incubation time 24 hr; concentration in nmoles/culture; mean ± SEM; number of experiments in parentheses.

[b]Significantly higher (Tukey-HSD test) than media alone.

[c]Significantly higher (Tukey-HSD test) than both media alone and highest TPA concentration.

TABLE 3

Effect of TPA and 10 μM IM on Prostanoid Synthesis (6-keto-$PGF_{1\alpha}$) and Lipid Peroxidation (MDA) in Confluent Cultures of SMC

TPA (M)	Incubation time 4 hr	24 hr
	6-keto-$PGF_{1\alpha}$	
0	0.009[a]	0.009
10^{-10}	0.009	0.024
10^{-9}	0.013	0.028
10^{-8}	0.012	0.030
10^{-7}	0.013	0.022
10^{-6}	0.014	0.032
	MDA	
0	0.11	0.48
10^{-10}	0.22	0.37
10^{-9}	0.22	0.32
10^{-8}	0.11	0.58
10^{-7}	0.27	0.37
10^{-6}	0.48	0.32

[a]Concentration in nmoles/culture; average of two determinations.

decreasing at all higher concentrations of TPA (Table 1). The prostanoid level in HNF cultures also was a function of the TPC concentration in the culture, again increasing to a maximum at 10^{-8}M TPA and decreasing at all higher concentrations of TPA (Table 2). Other investigators have shown that TPA stimulates the cyclooxygenase reaction (23-30). Our studies show that TPA has both a concentration-dependent stimulatory effect (low TPA concentration) and a concentration-dependent inhibitory effect (high TPA concentration) on prostanoid levels in SMC and HNF cell lines.

When cells were incubated with indomethacin (IM) in the 0.1 to 50 μM concentration range, prostanoid levels were decreased to a minimum with 10 μM and higher concentrations of IM. SMC were, therefore, incubated for 4 hr and 24 hr with 10 μM IM and increasing concentrations of TPA. These experiments showed that 10 μM IM abolished the stimulatory effect of TPA on prostanoid levels (Table 3). Similar data were obtained with random cultures of HNF where 10 μM IM diminished 6-keto-$PGF_{1\alpha}$ levels in the media by 96.8 ± 0.6%.

Since TPA enhances both fatty acid release from cellular phospholipids (23-26) and cyclooxygenase activity (23-30), the relative contribution of TPA to the two processes was estimated by incubating cells in the presence of a large excess of free 20:4(n-6) to minimize any TPA effect on fatty acid release. Prostanoid levels in SMC and HNF cultures incubated with 120 μM 20:4(n-6) and increasing concentrations of TPA again increased to a maximum at 10^{-8}M TPA (Table 4). At the TPA maximum (10^{-8}M TPA), the prostanoid increment (TPA/media in %) was much greater in media alone than in

TABLE 4

Effect of TPA and 120 μM 20:4(n-6) on Prostanoid Synthesis (6-keto-$PGF_{1\alpha}$) and Lipid Peroxidation (MDA) in Confluent Cultures of SMC and HNF

TPA (M)	SMC	HNF
	6-keto-$PGF_{1\alpha}$	
0	1.79 ± 0.25(8)[a]	1.69 ± 0.32(7)
10^{-10}	2.14 ± 0.21(4)	1.82 ± 0.31(7)
10^{-9}	3.31 ± 0.32(4)[b]	4.40 ± 0.49(7)[b]
10^{-8}	4.84 ± 0.20(8)[c]	7.52 ± 0.32(7)[b]
10^{-7}	3.91 ± 0.22(8)[c]	7.92 ± 0.56(7)[b]
10^{-6}	2.93 ± 0.15(8)[b]	7.07 ± 0.53(7)[b]
10^{-5}		7.26 ± 0.45(7)[b]
F ratio	27.57	38.97
	MDA	
0	14.37 ± 0.74(10)	7.26 ± 0.24(6)
10^{-10}	14.00 ± 0.79(4)	7.21 ± 0.46(7)
10^{-9}	13.83 ± 0.44(4)	7.53 ± 0.36(7)
10^{-8}	14.92 ± 0.72(10)	6.15 ± 0.22(7)
10^{-7}	13.89 ± 0.46(8)	6.47 ± 0.20(7)
10^{-6}	14.05 ± 0.42(8)	7.69 ± 0.40(7)
10^{-5}		6.79 ± 0.22(7)
F ratio	0.41	3.20

[a]Incubation time 24 hr; concentration in nmoles/culture; mean ± SEM; number of experiments in parentheses.

[b]Significantly higher (Tukey-HSD test) than media alone.

[c]Significantly higher (Tukey-HSD test) than both media alone and highest TPA concentration.

media containing 120 μM 20:4(n-6). Prostanoid increments for SMC were 680% (data from Table 1) and 270% (data from Table 4), respectively, and prostanoid increments for HNF were 1310% (data from Table 2), and 440% (data from Table 4), respectively. These data demonstrate the dual stimulatory effect of TPA on fatty acid release and cyclooxygenase activity when cells are incubated with TPA in media alone and the masking effect of exogenous 20:4 (n-6) on a prostanoid increment through fatty acid release.

Lipid Peroxides

Lipid peroxide levels in SMC cultures, measured as MDA, increased during a 24-hr incubation period. Data for 4 hr and 24 hr are reported in Table 1. The lipid peroxide level in SMC cultures was, unlike the prostanoid level, not a function of the TPA concentration in the media (Table 1). Similar results were obtained with HNF cultures in which increasing concentrations of TPA again had no effect on the lipid peroxide level (Table 2).

Since TPA stimulated the cyclooxygenase pathway, it was possible that lipid peroxide levels were not enhanced because the limited amount of available 20:4(n-6) was shunted to the cyclooxygenase pathway. This possibility was checked in two ways. In the first experiment, MDA levels were not enhanced when SMC were incubated with 10 μM IM and increasing concentrations of TPA (Table 3). In the second experiment, MDA levels were not enhanced when either SMC or HNF were incubated with 120 μM 20:4(n-6) and increasing concentrations of TPA (Table 4).

Previous investigators have shown that TPA stimulates lipoxygenase reactions (24,25,27-30). The absence of TPA effects on lipid peroxide levels when cells are incubated in media alone, media containing 10 μM IM, or media containing 120 μM 20:4(n-6) supports our previous observation that neither the lipoxygenase pathway nor co-oxidation during prostanoid synthesis are important in the generation of lipid peroxides in cultured SMC (14). The data also suggest that these specific peroxidation mechanisms are absent from cultured HNF.

Lipid Peroxidation and Prostanoid Synthesis

Although TPA had little effect on lipid peroxide levels measured as MDA, it was possible that lipid peroxides were formed and reduced by a peroxidase during the incubation period and that these peroxides modified the effect of TPA on the cyclooxygenase reaction. Since prostacyclin synthetase is particularly sensitive to lipid peroxides (9,13), 6-keto-$PGF_{1\alpha}$ levels were measured when SMC cultures were incubated with 10^{-8}M TPA, 120 μM 20:4(n-6), a mixture of TPA and 20:4(n-6), and two concentrations of the antioxidant α-tocopherol. The antioxidant blocked MDA formation in these cells but had no effect on 6-keto-$PGF_{1\alpha}$ levels (Table 5). Experiments with a second antioxidant, α-tocopherylquinone (5,7,9,12-15), showed that the antioxidant had no effect on the stimulation of either PGE_2 or 6-keto-$PGF_{1\alpha}$ synthesis when SMC cultures were incubated with 10^{-8}M TPA, 120 μM 20:4(n-6), or a mixture of TPA and 20:4(n-6) (Table 6). Previous studies from our laboratory had shown that guinea pig SMC synthesized similar amounts of PGE_2 and 6-keto-$PGF_{1\alpha}$ (8,14). These data were confirmed in cells stimulated with TPA in the α-tocopherylquinone experiments (Table 6).

Cell Proliferation

Cell growth in both SMC and HNF cultures was altered significantly by TPA. The cell number (Coulter Counter) in SMC cultures decreased to a minimum at 10^{-8}M TPA and then increased to the control level at higher TPA concentrations. Data with cells grown from one primary

TABLE 5

Effect of TPA and α-Tocopherol (E) on Prostanoid Synthesis (6-keto-$PGF_{1\alpha}$) and Lipid Peroxidation (MDA) in Confluent Cultures of SMC

Treatment[a]	6-keto-$PGF_{1\alpha}$	MDA
Media alone	0.31 ± 0.06(4)[b]	0.51 ± 0.05(4)
Media + 10 μM E	0.28(2)	0
Media + 50 μM E	0.20(2)	0
TPA	1.45 ± 0.12(4)	0.73 ± 0.06(4)
TPA + 10 μM E	1.99(2)	0.21(2)
TPA + 50 μM E	1.20(2)	0.10(2)
20:4(n-6)	2.03 ± 0.16(4)	12.79 ± 0.63(4)
20:4(n-6) + 10 μM E	2.31(2)	4.03(2)
20:4(n-6) + 50 μM E	1.69(2)	0.42(2)
TPA + 20:4(n-6)	5.43 ± 0.71(4)	10.70 ± 1.26(4)
TPA + 20:4(n-6) + 10 μM E	5.61(2)	6.00(2)
TPA + 20:4(n-6) + 50 μM E	5.00(2)	0.53(2)

[a]Incubations (24 hr) contained media alone, 10^{-8} M TPA, and 120 μM 20:4(n-6).

[b]Concentration in nmoles/culture; mean ± SEM; number of experiments in parentheses.

TABLE 6

Effect of TPA and α-Tocopherylquinone (EQ) on PGE_2 and 6-keto-$PGF_{1\alpha}$ Synthesis in Confluent Cultures of SMC

Treatment[a]	PGE_2	6-keto-$PGF_{1\alpha}$
Media	0.40[b]	0.35
Media + EQ	0.40	0.44
TPA	2.56	2.20
TPA + EQ	2.91	3.18
20:4(n-6)	3.27	3.65
20:4(n-6) + EQ	3.27	4.53
TPA + 20:4(n-6)	14.82	8.11
TPA + 20:4(n-6) + EQ	14.82	7.61

[a]Incubations (24 hr) contained media alone, 50 μM EQ, 10^{-8} M TPA, and 120 μM 20:4(n-6).

[b]Concentration in nmoles/culture; average of two determinations.

culture are reported in Table 7. Similar data, obtained with cells grown from two other primary cultures, showed a maximum decrease in cell number that averaged 30% at 10^{-8}M TPA. HNF were even more susceptible than SMC to the inhibitory effect of TPA on cell growth (Table 7). Cell number again decreased to a minimum in the 10^{-9} to 10^{-8}M TPA concentration range. The decrease in this range was as high as 72% and, although growth increased at higher TPA concentrations, HNF growth did not approach control levels.

Since 10 μM IM blocked prostanoid synthesis in cell cultures, SMC and HNF cultures were grown in 10 μM IM and increasing amounts of TPA. IM had no effect on the inhibition of growth in either cell line (Table 7) even though 10 μM IM blocked prostanoid synthesis and probably raised the level of free 20:4(n-6). Other preliminary studies also showed that TPA and 90 μM 20:4(n-6) did not have a synergistic inhibitory effect on cell growth.

SMC were grown in cultures with 10 μM butylated hydroxytoluene (BHT) and increasing amounts of TPA. BHT, an antioxidant that blocks lipid peroxidation (5,9,14), had no effect on cell growth which again decreased to a minimum with 10^{-8}M TPA (Table 8). Similar data were obtained with another antioxidant, dipyridamole (9,12), when cells were grown in 1 μM dipyridamole and increasing amounts of TPA.

Because the decrease in cell number may have reflected either an increase in the population doubling time or a decrease in the number of colonies (cytotoxicity), image analysis was used to count the number of colonies growing in SMC cultures treated with TPA. These data showed that total colony number decreased to a minimum with 10^{-8}M TPA and then increased with 10^{-6}M TPA (Fig. 1). Thus cell growth followed cytotoxicity.

DISCUSSION

The concentration-dependent stimulatory (low TPA levels) and inhibitory (high TPA levels) effects of TPA on prostanoid synthesis are found in both SMC and HNF (Tables 1 and 2). The explanation of these phenomena may be found in cellular oxidation reactions. Prostanoid synthesis is stimulated by low concentrations of oxidants such as hydrogen peroxide (45,46) and activator lipid peroxides (47-49), and prostanoid synthesis is blocked by high concentrations of oxidants that inactivate the PGH synthase complex (47-49). This apparent paradox explains why antioxidants in low concentrations protect the synthase and stimulate prostanoid synthesis, and why antioxidants in high concentrations destroy activator oxidants and block prostanoid synthesis (50-53). Oxidant effects explain why the organic hydroperoxide t-butyl hydroperoxide stimulates prostanoid synthesis when it is added to cells in low concentrations and inhibits prostanoid synthesis when its concentration is increased (54). TPA stimulates a receptor-mediated metabolic burst in cells generating hydrogen peroxide and oxygen-centered radicals such as superoxide (13,

TABLE 7

Effect of TPA on Cell Number (Coulter Counter) of SMC and HNF Cultures Grown in the Presence and Absence of 10 μM IM

TPA (M)	Cell number (-)IM	Cell number (+)IM
	SMC	
0	356,000 ± 14,000(7)[a]	325,000 ± 8,210(5)
10^{-10}	282,000 ± 9,390(8)[b]	282,000 ± 10,600(7)
10^{-9}	310,000 ± 15,700(6)	317,000 ± 15,200(6)
10^{-8}	242,000 ± 5,960(6)[c]	247,000 ± 9.770(7)[c]
10^{-7}	322,000 ± 16,900(6)	296,000 ± 10,400(8)
10^{-6}	350,000 ± 17,500(8)	310,000 ± 12,300(8)
F ratio	9.22	5.98
	HNF	
0	67,800 ± 2,760(6)	82,200 ± 2,850(8)
10^{-10}	30,500 ± 1,840(7)[b]	42,300 ± 1,480(7)[b]
10^{-9}	18,400 ± 1,340(8)[c]	22,300 ± 1,330(8)[c]
10^{-8}	19,200 ± 516(8)[c]	22,200 ± 2,132(8)[c]
10^{-7}	28,000 ± 534(8)[c]	30,300 ± 3,440(5)[b]
10^{-6}	34,600 ± 1,550(6)[b]	33,500 ± 3,220(6)[b]
F ratio	137.2	99.66

[a]Mean ± SEM; number of experiments in parentheses.

[b]Significantly lower (Tukey-HSD test) than media alone.

[c]Significantly lower (Tukey-HSD test) than both media alone and highest TPA concentration.

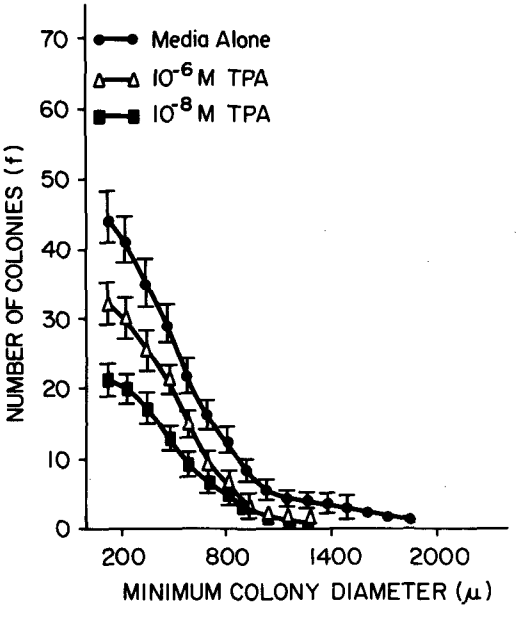

FIG. 1. Colony frequency-size distribution for SMC grown in Experimental Media alone or media containing TPA. The number of colonies was obtained by image analysis. The total colony number was obtained from the frequency at the lowest minimum colony diameter. Frequency data are displayed as the complement of a cumulative distribution function. Vertical lines show SEM.

TABLE 8

Effect of TPA on Cell Number (Coulter Counter) of SMC Cultures Grown in the Presence and Absence of 10 μM BHT

TPA (M)	Cell number (-) BHT	Cell number (+) BHT
0	95,900 ± 972(8)[a]	132,000 ± 4,220(8)
10^{-10}	93,000 ± 1,620(7)	108,000 ± 4,070(8)[b]
10^{-9}	62,400 ± 2,380(8)[c]	78,400 ± 1,830(8)[b]
10^{-8}	60,600 ± 1,720(8)[c]	72,600 ± 2,400(8)[c]
10^{-7}	77,400 ± 2,770(8)[b]	80,800 ± 1,760(8)[b]
10^{-6}	86,200 ± 5,410(8)	88,200 ± 2,410(8)[b]
F ratio	27.20	58.33

[a]Mean ± SEM; number of experiments in parentheses.

[b]Significantly lower (Tukey-HSD test) than media alone.

[c]Significantly lower (Tukey-HSD test) than both media alone and highest TPA concentration.

16-22). Oxidant levels vary directly with the TPA concentration (21,22). Low concentrations of TPA evidently generate stimulatory oxidant levels while high concentrations of TPA evidently generate inhibitory oxidant levels for prostanoid synthesis in SMC and HNF cultures (Tables 1 and 2).

There is little evidence in the present study that TPA has any effect in SMC and HNF cul-

tures on lipid peroxidation measured as MDA. This conclusion is supported by incubation studies in the absence of IM (Tables 1 and 2), incubation studies in the presence of IM (Table 3) and incubation studies in the presence of an excess 20:4(n-6) substrate concentration (Table 4). Experiments with the antioxidants α-tocopherol and α-tocopherylquinone (Tables 5 and 6) show that activator lipid peroxides are not involved in the stimulation of prostanoid (PGE_2 and 6-keto-$PGF_{1\alpha}$) synthesis with TPA, since lipid peroxides are destroyed by these antioxidants and yet the antioxidants have little effect on prostanoid levels. TPA evidently stimulates prostanoid synthesis through the generation of water soluble oxygen centered radicals.

Many studies link the function of TPA as a tumor promoter to its effects on 20:4(n-6) metabolism through both the cyclooxygenase and lipoxygenase pathways (24,25,27-30,36-41). TPA has pleiotropic effects on a variety of cellular events, and it is difficult to separate concurrent effects from other effects that may have a causal relationship. The problem is demonstrated by our studies on 20:4(n-6) metabolism and cell proliferation in SMC and HNF.

Experiments summarized in Figure 2 show that the maximum increment in the prostanoid level occurs at the same TPA concentration in SMC and HNF. The increment is much greater in HNF, and the prostanoid level in this cell line does not return to the baseline value at a high TPA concentration. Experiments summarized in Figure 3 show that the maximum decrease in cell growth with SMC and HNF occurs in the same TPA concentration range as the maximum prostanoid increment. Furthermore, the decrease in cell growth is much greater with HNF, and cell growth in these cultures does not return to the baseline value at a high TPA concentration. Prostanoids in high concentrations inhibit cell growth (3,12,13), and the studies summarized here seem to link high prostanoid levels with diminished cell growth. However, IM blocks prostanoid synthesis and as a consequence probably raises the level of free 20:4 (n-6) without altering cell growth (Table 7). Lipid peroxidation often is invoked as an aspect of 20:4(n-6) metabolism that explains the absence of an IM effect (13,28-30,41). However, antioxidants such as BHT block lipid peroxidation without altering cell growth (Table 8). Thus, TPA diminished cell growth through a cytotoxic effect (Fig. 1) that probably is unrelated to 20:4(n-6) metabolism. Suggestions (14, 16,24,25,28-30,36-41) that TPA acts through

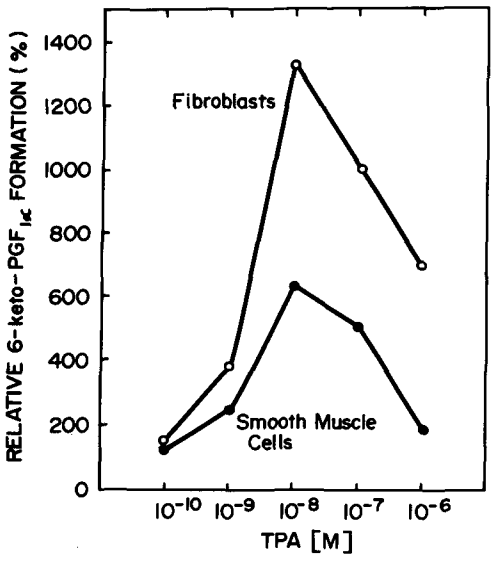

FIG. 2. Relative 6-keto-$PGF_{1\alpha}$ levels (TPA/media in %) for SMC and HNF (fibroblasts) as a function of the TPA concentration. Values were calculated from data in Tables 1 and 2.

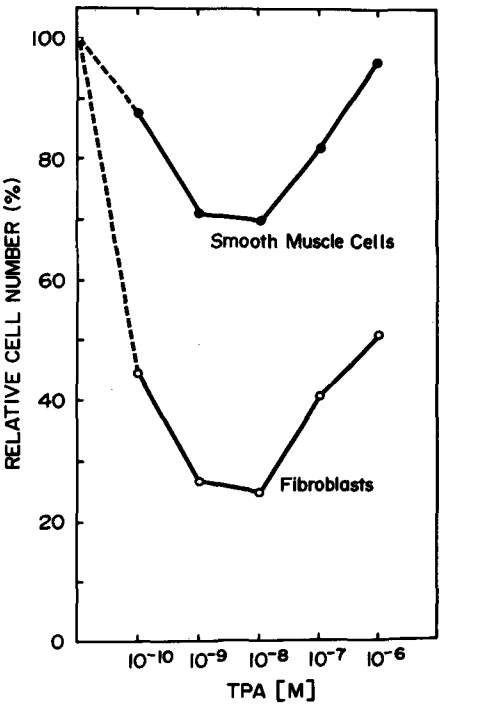

FIG. 3. Relative cell number (TPA/media in %) for SMC and HNF (fibroblasts) as a function of the TPA concentration. Values were calculated from data in Table 7.

the 20:4(n-6) cascade and associated lipid peroxidation reactions in tumor promotion and the regulation of cell growth deserve further explanation.

ACKNOWLEDGMENTS

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METHODS

High Performance Liquid Chromatographic Separation of Diacylglycerol Acetates to Quantitate Disaturated Species of Lung Phosphatidylcholine

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ABSTRACT

A high-performance liquid chromatographic (HPLC) separation of diacylglycerol acetates to quantitate disaturated species of lung phosphatidylcholine (PC) was studied. The diacylglycerol acetates were applied on a reversed phase column, eluted by an isocratic solvent, acetonitrile/isopropanol/water (35:15:1, v/v/v) at a flow rate of 1 ml/min, and detected by differential refractometry (RI). This isocratic HPLC method was useful to separate disaturated species from the others of lung PC.

The quantitative analysis of the molecular species separated by HPLC was studied by RI detection. Chromatograms obtained by RI detection and radioactivity determination of diacylglycerol [^{3}H] acetates prepared by [^{3}H] acetic anhydride were almost identical. The RI detector responsed in the same degree for different authentic standards of diacylglycerol acetates. The detection limit with RI detection was about 30 nmoles. Molecular species of PCs from human lung and carcinoma tissues were analyzed by this HPLC method. The contents of disaturated species were very similar to those reported previously. These results indicate that RI detection is very useful in the nmole range for the quantitative analysis among the molecular species containing disaturated species.
Lipids 20:611-616, 1985.

INTRODUCTION

Several methods for resolution of lung phospholipids into the molecular species have been presented, i.e., $AgNO_3$-thin layer chromatography (TLC) (3,4), the combinations of $AgNO_3$-TLC and gas liquid chromatography (GLC) (5, 6), and GLC-mass spectrometry (7). Recently, high performance liquid chromatographic (HPLC) methods for the separation of molecular species of phospholipids have been reported in several mammalian tissues (8-12), which do not contain appreciable amounts of disaturated species. However, there has been no report published regarding HPLC separation and quantitative analysis of lung phospholipids containing large amounts of disaturated species. In this communication, a rapid, isocratic HPLC method for the separation of disaturated species from others of lung PC is presented, and the differential refractometric detection is examined for the quantitative analysis of individual molecular species of lung PC.

MATERIALS AND METHODS

Materials

Wistar rats weighing 250 g, maintained on a diet (Oriental Co., Japan) (13), were used. Human normal lung and carcinoma tissues were obtained at the time of operation in Sapporo Medical College Hospital, Japan. The standards of phosphatidylcholine (PC) molecular species, 1,2-dimyristoyl, 1,2-dipalmitoyl, 1-palmitoyl-2-oleoyl and 1-palmitoyl-2-linoleoyl PCs were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). High performance liquid chromatographic grade organic solvents and water were purchased from Katayama Chemical Co. (Japan).

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Preparation of Diacylglycerol Acetates from Phosphatidylcholines

Lipids were extracted from rat lung, human lung and human carcinoma tissues by the methods of Folch et al. (14). PC was isolated preparatively by TLC using silica gel G plates with a solvent system of chloroform/methanol/conc. ammonia/water (70:30:3:2, v/v/v/v). After detection with a fluorescein reagent, the PC spot on the plates was scraped off and eluted with chloroform/methanol/acetic acid/water (50:39:1:10, v/v/v/v) as described by Arvidson (15), and the extract was washed with 4N ammonia and 50% methanol in saline. The isolated PC gave a single spot on two-dimensional thin layer chromatography as described by Poorthuis et al. (16).

The PCs were converted into diacylglycerols by phospholipase C from *Clostridium perfringens* (Sigma Chemical Co., St. Louis, Missouri, USA) (17). The diacylglycerols were acetylated immediately with acetic anhydride and anhy-

drous pyridine (18). The diacylglycerol acetates were then purified by TLC with a solvent system of hexane/ether/formic acid (60:20:1.5, v/v/v) and recovered by the method of Arvidson (18). The yield of diacylglycerol acetate from PC in this procedure was about 80% when examined using [^{14}C]dipalmitoyl phosphatidylcholine (Amersham, United Kingdom). When [^{3}H]acetic anhydride was used, the acetylation was carried out according to the procedure of Ishidate and Weinhold (19). The purified diacylglycerol acetates were dissolved in aliquots of acetonitrile/isopropanol/water (35:15:1, v/v/v) and applied to the column.

High-Performance Liquid Chromatography (HPLC)

The HPLC separations were carried out on a BAS liquid chromatographic system equipped with a variable wavelength detector (model UVITEC-100, Nihon Bunko Co., Japan), which was operated at 205 nm, or a differential refractometer (RI detector, × 8) (model ERC-7520, Erma Co., Japan) in conjunction with an integrator (Shimidzu Chromatopac 51A). The diacylglycerol acetates were separated by chromatography on a 250 × 4.6 mm Hibar II column packed with LiChrosorm RP-18 (10 μm) (Merck, Darmstadt, Federal Republic of Germany). The eluting solvent system was an isocratic solvent, acetonitrile/isopropanol/water (35:15:1, v/v/v), pumped at a flow rate of 1 ml/min at room temperature. When each fraction was collected, the flow rate was 0.2 ml/min.

Gas-Liquid Chromatography

Fractions from the column were collected for the identification and quantitation of each peak by GLC. Fatty acids from diacylglycerol acetates were determined at 200 C using a Model 5A gas chromatograph (Shimadzu Co., Japan) equipped with a pyrex column (1.5 m × 4 mm) packed with Unisol 3000 (Uniport C, Shimadzu Co., Japan), after the methylation with BF_3/methanol (20). In order to determine the total acyl carbon number, diacylglycerol acetates also were analyzed by GLC using a pyrex column (1.5 m × 4 mm) with 1% silicone OV-1 on Gaschrom Q at 230 C.

Other Analytical Methods

Phosphorus was determined by the method of Bartlett (21). The amount of diacylglycerol acetates was estimated by glycerol determination according to the method of Van Handel and Zilversmit (22). Radioactivity determination was made with a Beckman LS-9000 scintillation spectrometer using ACS II scintillator (Amersham, United Kingdom).

RESULTS AND DISCUSSION

Sixteen peaks were detected on the chromatograms of the diacylglycerol moieties of PC from rat lung by UV and RI detections (Fig. 1). The molecular species contained in each peak were identified by the analyses of the total fatty acids and the total acylcarbon number of diacylglycerol acetates using GLC. Results of the analyses are shown in Table 1. Nine peaks, i.e., peaks 4, 7, 8, 10, 11, 12, 14, 15 and 16, were observed as peaks containing mostly a single molecular species. The main molecular species in each peak are illustrated in Table 2. The tendency for the separation of the diacylglycerol acetates by the reversed column was similar to those findings reported previously (8,9). The retention time of each molecular species was longer with increasing chain length and shorter with increasing degree of the unsaturation. That is, polyunsaturated species were eluted earlier than oligoenoic species. Peaks 1 to 4, which were not significant peaks in lung PC, seemed to be composed of various polyunsaturated species. Peaks 9 and 13 were mixtures of monoenoic and dienoic species. However, disaturated species, i.e., 14:0/16:0, 16:0/16:0 and 16:0/18:0 species, which are characteristic molecular species of lung PC, were separable from the others as a single peak. This isocratic HPLC method is certainly useful to separate disaturated species of lung PC.

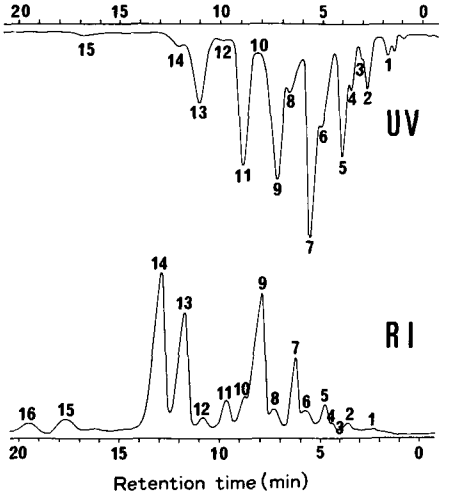

FIG. 1. High performance liquid chromatographic separation of diacylglycerol acetates derived from rat lung phosphatidylcholine. Detection: upper chromatogram, UV absorption at 205 nm; lower chromatograms, refractometry detection (RI × 8).

TABLE 1

Composition of Fatty Acid and Total Acyl Carbon Number in Each Peak Obtained by High Performance Liquid Chromatography of Diacylglycerol Acetates Derived from Rat Lung Phosphatidylcholine

	Peak number[a]															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Fatty acid (% in each peak)																
14:0	6.3	–	13.1	–	1.5	–	–	0.8	–	31.4	2.1	0.6	–	–	–	–
16:0	4.3	17.7	29.3	37.2	28.7	26.1	46.5	2.5	48.2	53.6	9.6	4.2	35.7	98.8	8.3	36.7
16:1	–	5.7	12.3	–	13.9	1.1	–	–	12.6	–	1.9	8.0	–	–	6.9	–
18:0	5.8	0.8	–	9.4	0.7	–	0.2	–	–	6.3	45.0	–	14.1	1.0	48.0	46.5
18:1	5.7	16.3	–	5.2	0.6	12.2	0.3	54.6	3.8	–	–	71.0	38.8	–	36.1	14.7
18:2	15.9	24.2	27.1	–	23.1	1.1	1.3	37.9	30.7	4.6	2.6	10.8	11.4	0.2	0.7	2.1
18:3	–	–	–	–	–	6.7	–	–	–	–	–	–	–	–	–	–
20:3	3.4	3.8	–	–	–	1.6	–	2.8	4.7	4.1	–	4.6	–	–	–	–
20:4	42.4	–	–	–	4.6	10.0	50.6	0.6	–	–	38.8	–	–	–	–	–
20:5	7.2	31.5	18.2	48.2	1.5	–	–	0.8	–	–	–	0.8	–	–	–	–
22:5	4.9	–	–	–	–	27.3	–	–	–	–	–	–	–	–	–	–
22:6	4.1	–	–	–	24.8	13.9	1.1	–	–	–	–	–	–	–	–	–
Total acyl carbon number[b] (% in eack peak)																
30	–	–	–	–	–	–	–	–	–	93.2	8.6	–	–	–	–	–
32	1.7	2.3	–	18.7	16.0	–	–	–	31.5	4.4	2.2	18.7	–	98.6	4.0	5.7
34	21.3	27.4	74.7	–	23.0	8.1	–	17.4	64.4	2.4	4.2	–	79.5	1.4	11.4	79.5
36	23.9	24.3	14.2	81.3	25.3	21.4	98.2	82.6	2.4	–	1.8	81.3	20.5	–	81.6	10.8
38	45.7	46.0	11.1	–	35.7	70.5	0.8	–	1.7	–	83.0	–	–	–	3.0	3.9
40	7.4	–	–	–	–	–	1.0	–	–	–	–	–	–	–	–	–

[a]Peak numbers are shown in Figure 1.

[b]Total acyl carbon numbers represent total carbon numbers of fatty acids at 1- and 2-position.

TABLE 2

Main Molecular Species in Each Peak, Determined by Analyses of Fatty Acids and Total Acyl Carbon Numbers

Peak number[a]	Main molecular species			
1	18:2 / 20:4 [b]			
2	18:1 / 20:5			
3	16:0 / 20:5			
4	16:0 / 20:5	(81%)		
5	16:0 / 22:6	(36%)	16:1 / 18:2	(23%)
6	18:1 / 18:3	(21%)	16:0 / 22:6	(70%)
7	16:0 / 20:4	(98%)		
8	18:1 / 18:2	(83%)		
9	16:0 / 18:2	(64%)	16:0 / 16:1	(31%)
10	14:0 / 16:0	(93%)		
11	18:0 / 20:4	(83%)		
12	18:1 / 18:1	(81%)		
13	16:0 / 18:1	(79%)	18:0 / 18:2	(20%)
14	16:0 / 16:0	(99%)		
15	18:0 / 18:1	(82%)		
16	16:0 / 18:0	(80%)		

[a]Peak numbers are shown in Figure 1.

[b]Values in parenthesis represent percent distributions of main molecular species in each peak.

As shown in Figure 1, the chromatogram tracing obtained by UV detection at 205 nm was significantly different from that obtained by RI detection. Marked differences were obtained in peaks containing disaturated species, peaks 10, 14 and 16. These peaks were very small by the UV detection, but were significant by the RI detection. It is well known that UV absorption at 205 nm varies with the degree of unsaturation. The disaturated species, which do not have any double bond in the fatty acid chains, showed only a little UV absorption, which might depend on carbonyl bonds in the molecules. Present results indicated that the peak areas obtained by UV absorption cannot be used as a basis of quantitation, especially for the disaturated species.

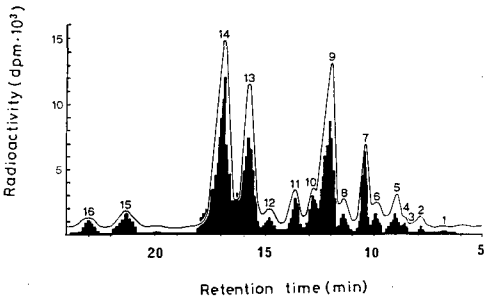

FIG. 2. Comparison of refractometry detection and radioactivity distribution in high performance liquid chromatographic separation of diacylglycerol [^{3}H]acetates derived from rat lung phosphatidylcholine. Diacylglycerol [^{3}H]acetates were prepared chemically using [^{3}H]acetic anhydride. Line tracing represents a chromatogram obtained using RI detection. Stepwise tracing (black area) represents distribution of [^{3}H]radioactivity collected in a constant interval.

The diacylglycerol [^{3}H]acetates were prepared from lung PC using [^{3}H]acetic anhydride. Since all the molecular species of diacylglycerol acetates are labeled equally by the [^{3}H]radioactivity, the radioactivity distribution in the chromatogram of the diacylglycerol [^{3}H]acetates represents a mass distribution of the molecular species. Figure 2 shows a comparison of the chromatograms obtained by RI detection and the radioactivity distribution of the molecular species of diacylglycerol [^{3}H]acetates from rat lung PC. The chromatograms were almost identical. Table 3 shows the quantitative data of the two chromatograms. The percent compositions among peaks obtained by the RI detection is a measure of the mass distribution of the molecular species of diacylglycerol acetates.

The RI detector responses were examined for different diacylglycerol acetates prepared from commercial standards of PC. As shown in Figure 3, the dose-response curves were superimposable for different molecular species of diacylglycerol acetates, indicating that RI detection dose not discriminate the degree of unsaturation and the chain length of the diacylglycerol acetates. Therefore, Figure 3 can be used as a standard curve for the quantitation of all diacylglycerol acetates. The RI detection was sensitive in the range of 20-150 μg of the diacylglycerol acetates. The detection limit was 20 μg for dipalmitoylglycerol acetate, which corresponds to about 30 nmoles.

TABLE 3

Comparison of Molecular Species Compositions Obtained by Refractometry Detection and Radioactivity Determination of Diacylglycerol [^{3}H]acetates Derived from Rat Lung Phosphatidylcholines by High Performance Liquid Chromatography

Peak number[a]	Percent distribution (%)	
	Refractometry detection (n=4)	Radioactivity (n=3)
1	tr[b]	tr
2	0.5 ± 0.1	0.7 ± 0.1
3	0.1	0.3
4	0.5 ± 0.2	0.4 ± 0.1
5	3.0 ± 0.3	2.6 ± 0.5
6	2.8 ± 0.2	3.6 ± 0.3
7	7.5 ± 0.2	7.4 ± 0.1
8	3.1 ± 0.2	2.9 ± 0.1
9	21.3 ± 0.4	21.5 ± 0.3
10	4.4 ± 0.2	5.0 ± 0.3
11	4.5 ± 0.2	5.6 ± 0.1
12	2.1 ± 0.1	2.5 ± 0.1
13	18.7 ± 0.4	16.7 ± 0.4
14	26.8 ± 0.6	27.0 ± 0.6
15	3.0 ± 0.5	2.8 ± 0.1
16	1.7 ±0.2	1.0 ± 0.1

[a]Peak numbers are shown in Figure 1.

[b]tr: less than 0.05%.

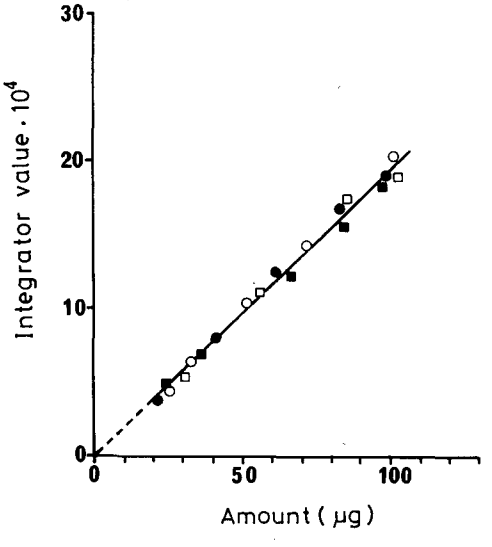

FIG. 3. Responses by refractometry detection for different amounts of each molecular species of diacylglycerol acetates in high performance liquid chromatography. •, dipalmitoyl species; ○, dimyristoyl species; ■, palmitoyloleoyl species; □, palmitoyl-linoleoyl species.

These findings demonstrate clearly that it is possible to quantitate molecular species of diacylglycerol acetates derived from lung PC by HPLC using RI detection. Since the disaturated species, such as 14:0/16:0, 16:0/16:0 and 16:0/18:0 species, can be separated as a single peak by the reversed column, it is possible to determine them quantitatively when the RI detector is used. As shown in Table 3, the contents of disaturated species of rat lung PC are 4.4% for 14:0/16:0 species, 26.8% for 16:0/16:0 species and 1.7% for 16:0/18:0 species, respectively. These values are in good agreement with those reported by Okano et al. (5), who analyzed molecular species of rat lung PC using $AgNO_3$-TLC of the diacylglycerol acetates followed by gas chromatography.

p-Nitrobenzoyl derivatives of diacylglycerols from rat lung PC, which were expected to be more sensitive, were examined at 290 nm of UV absorption. However, considerable differences were observed between the chromatograms obtained using the derivatives and the radioactivity distribution of diacylglycerol [^{3}H]acetates, especially in the arachidonyl species (data not shown). The detection limit of the derivatives at 290 nm was not as high as expected, which was also in the nmole range. It should be used carefully to quantitate the UV sensitive derivatives of diacylglycerols, because UV absorption varies with the degree of unsaturation of fatty acid chains.

Compositions of molecular species of PC from human lung and carcinoma tissues were determined by HPLC using RI detection. Results are shown in Table 4. The content of the disaturated species in PC of normal human lung was 32.2%, i.e., 6.2% for 14:0/16:0 species, 24.8% for 16:0/16:0 species and 1.2% for 16:0/18:0 species, but the contents in lung adenocarcinoma and squamous cell carcinoma were 15.9% and 12.1%, respectively. The disaturated species in stomach carcinoma was significantly lower than in the lung carcinomas. These data were similar to those obtained using $AgNO_3$-TLC, which we have reported previously (23,24).

We reported that the quantitation of disaturated species of PC, particularly dipalmitoyl species, is very useful for the differentiation of lung adenocarcinoma originating from alveolar type II cells (25). It also will be useful for the differentiation between a metastatic and a primary adenocarcinoma in the lung, which has been difficult and in some cases impossible for pathologists to diagnose (23). The HPLC method reported here is a more simple and rapid procedure than those reported previously for the analysis of disaturated species of PC.

TABLE 4

Molecular Species Composition of Phosphatidylcholine of Various Tissues Determined by High Performance Liquid Chromatography

Peak number[b]	Molecular species composition (%)[a]			
	Human lung (n=3)	Human lung adenocarcinoma (n=3)	Human lung squamous cell carcinoma (n=2)	Human stomach adenocarcinoma (n=3)
1	tr[c]	tr	tr	tr
2	tr	tr	tr	tr
3	tr	tr	tr	0.1 ± 0.2
4	1.2 ± 0.9	1.0 ± 0.4	0.8	0.2 ± 0.3
5	1.8 ± 0.6	2.7 ± 0.3	2.5	3.5 ± 0.6
6	1.4 ± 0.4	1.3 ± 0.4	3.1	2.9 ± 1.2
7	3.3 ± 1.4	5.2 ± 1.6	6.8	11.5 ± 3.2
8	4.9 ± 1.5	5.0 ± 1.7	6.5	6.6 ± 1.3
9	20.0 ± 0.9	24.1 ± 3.0	14.7	19.9 ± 2.1
10	6.2 ± 1.5	2.4 ± 0.2	1.7	tr
11	3.3 ± 0.8	4.5 ± 1.0	5.6	7.2 ± 0.9
12	3.7 ± 1.4	2.7 ± 2.4	9.4	3.6 ± 0.2
13	25.1 ± 0.8	33.1 ± 2.3	30.9	29.2 ± 3.5
14	24.8 ± 3.5	13.5 ± 2.4	9.0	7.4 ± 0.3
15	3.8 ± 0.3	4.5 ± 0.7	6.6	5.6 ± 1.8
16	1.2 ± 0.2	–	1.4	0.8 ± 0.7

[a]Main molecular species in each peak are shown in Table 2.

[b]Peak number is shown in Figure 1.

[c]tr: less than 0.05%.

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The Effect of Fatty Acid Chain Length and Unsaturation on the Chromatographic Behavior of Triglycerides on Iatroscan Chromarods

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ABSTRACT

The chromatographic behavior of lipid classes on Chromarods with common developing solvents used for the Iatroscan system was influenced by the esterified fatty acids present in the lipid class. The R_f value of the lipid class increased with increasing chain length and increasing unsaturation of the fatty acids; the increase in R_f value per methylene carbon was about twice that per double bond. The effect was evident in both triglycerides and phosphatidylcholines. This partial separation of molecular species within a lipid class caused widening of the lipid class band on the Chromarods and can result in poorer resolution, particularly in biological samples.
Lipids 20:617-619, 1985.

INTRODUCTION

Thin layer chromatography (TLC) using silica gel is an effective method of separating lipids into compound classes, generally without regard to fatty acid chain length and unsaturation (1,2). To separate members according to either chain length or unsaturation involves impregnating the adsorbent respectively with silicones or paraffins, or silver nitrate. These same principles also were assumed to apply to the separation of lipids on Chromarods used in the Iatroscan instrument. Chromarods are quartz rods coated with silica gel held in place with a special frit of soft glass (3). In our studies we repeatedly observed that standard neutral lipid mixtures containing a single fatty acid (4) give sharper peaks than the same lipid class from a biological sample (5). In biological samples the triglycerides (TGs) and phospholipids are a complex mixture of fatty acids among the species of glycerols within each lipid class. The fatty acids may range in chain length from 14-24 and have up to three double bonds in plant lipids and up to six double bonds in animal lipids. Here we report the effect of different fatty acids in a lipid class on their chromatographic behavior on Chromarods using common developing solvents.

MATERIALS AND METHODS

Pure TG (>99%) of 16:1 n-7, 22:1 n-9 and 22:6 n-3, and methyl heptadecanoate were obtained from Nu-Chek-Prep (Elysian, Minnesota, U.S.A.); 1,2-dipalmitoyl phosphatidylcholine (PC) was obtained from Applied Science (State College, Pennsylvania, U.S.A.). The following mixtures were prepared: methyl heptadecanoate with each of the three pure TGs, methyl heptadecanoate with all three combinations of any two of the TGs, and methyl heptadecanoate with any equal mixture of all three of the TGs. The ratio of methyl ester (internal standard) to total TG was ca 1:1. These lipid mixtures were dissolved in heptane.

A new set of 10 Chromarods (type S) was acid washed in $H_2SO_4/HClO_4$ (1:1, v/v), rinsed in distilled water and burned three times before use. One μl of solution, containing ca 4 μg of the methyl ester and ca 4 μg total TG, was spotted onto the clean Chromarods and developed in 1,2-dichloroethane/chloroform/acetic acid (92:8:0.1, v/v/v) (6). After development, the Chromarods were dried for 2 min at 80 C and scanned on the Iatroscan as described previously (4,5). The phospholipids from rat heart (5) and dipalmitoyl PC were dissolved in chloroform/methanol (1:1, v/v), spotted, and developed twice to a height of 10 cm in chloroform/methanol/water (68.5:29:2.5, v/v/v) (5).

RESULTS AND DISCUSSION

The TGs of 16:1 n-7, 22:1 n-9 and 22:6 n-3 were selected to maximize differences in fatty acid chain length and unsaturation which might be found when biological samples are analyzed. It is evident from Figure 1 that these three TGs partially separated on Chromarods. The R_f value of the TG increased with its fatty acid chain length (16:1 n-7 vs 22:1 n-9) and its unsaturation (22:1 n-9 vs 22:6 n-3). The average R_f increment from four analyses per rod and 10 rods per set was R_f 0.082±0.008 for 18 methylene carbons (difference between 16:1 n-7 TG and 22:1 n-9 TG), or R_f 0.0046 per methylene carbon, and R_f 0.034±0.003 for 15 double bonds (difference between 22:1 n-9 TG and 22:6 n-3 TG), or R_f 0.0023 per double bond.

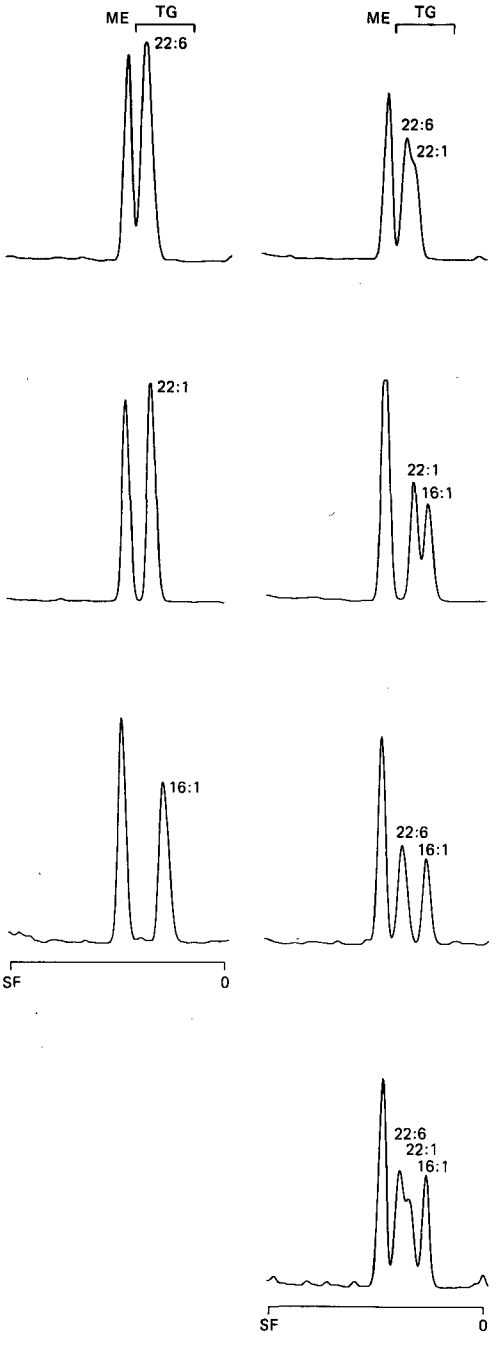

FIG. 1. Separation on Chromarods of triglycerides (TG) containing either 16:1 n-7, 22:1 n-9 or 22:6 n-3 fatty acids with methyl heptadecanoate (ME) as internal standard. Pure triglycerides (left side), and mixtures of any two (right side) and all three (bottom) triglycerides were analyzed. O = origin, SF = solvent front.

The partial separation of molecular species within the TG class helps explain the size and shape of this peak in biological samples. Sipos and Ackman (7), for example, published chromatograms of herring oil, northern quahaug and monkey serum lipids. In each case, the shape of the TG peak was very broad and skewed towards higher R_f values, which is to be expected because all these TGs have a wide spread in fatty acid chain lengths and are rich in long chain, highly unsaturated fatty acids. On the other hand, the shape of the tristearin peak was fairly narrow and symmetrical (7).

The partial separation of lipid classes depending on their fatty acid composition applies not only to TG, but also to polar lipids such as PC. This was demonstrated in the following manner. A typical separation of rat heart polar lipids (Fig. 2, top chromatogram) shows a symmetrical peak for PC (insert). When this heart lipid sample was spiked with 1,2-dipalmitoyl PC (Fig. 2, bottom chromatogram), the mixture clearly showed that the shorter chain saturated PC migrated on the lower side (smaller R_f value) of the PC peak. Furthermore, 1,2-dipalmitoyl PC appeared to widen the cardiac PC peak as evidenced by comparing PC in the top and bottom chromatograms in Figure 2.

A partial separation among both neutral triacyl (TG) and polar diacyl (PC) lipids differing in their fatty acid chain length and unsaturation appeared to be a major reason (apart from poor spotting techniques) for the generally wider band width for lipid classes of natural origin and resultant poorer resolution of lipids from biological origin on Chromarods (5,8-10). Publications in which chromatograms of standard lipid mixtures appear show generally sharper, better resolved peaks, particularly when examining compounds migrating in close proximity (4,6,11-13). The difference in resolution was particularly evident in publications showing chromatograms of both standards and biological samples and appears to apply to both type S and SII Chromarods (7,14,15).

The results of this study help explain why the resolution of lipid classes in samples of biological origin is not as good as in standard mixtures containing one kind of acyl group. We reported previously the difficulty of resolving phosphatidylethanolamine and phosphatidylinositol from rat heart lipids (5). The wide spread in chain length and unsaturation of fatty acids in these two phospholipids (16) could be one reason why these peaks were poorly resolved.

The analytical methods described here obviously do not provide satisfactory means of separating lipid classes based on differences in chain length and unsaturation of the fatty acid

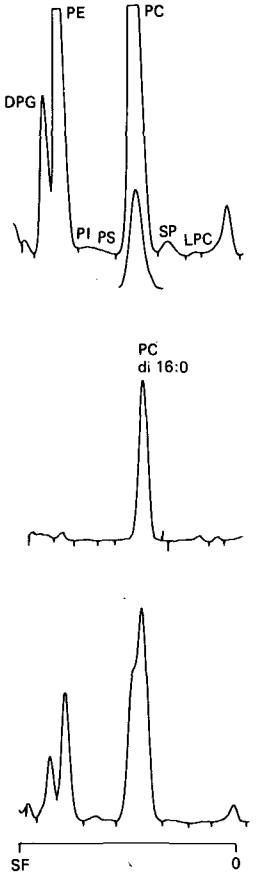

FIG. 2. Separation on Chromarods: of rat heart polar lipids (top chromatogram) with an insert of phosphatidylcholine (PC) obtained by reducing the attenuation; of 1,2-dipalmitoyl (di 16:0) PC (center chromatogram), and of rat heart polar lipids spiked with di 16:0 PC (bottom chromatogram). DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SP, sphingomyelin; LPC, lysophosphatidylcholine. O = origin, SF = solvent front.

moieties. Separations based on difference in fatty acid unsaturation can be achieved more effectively by impregnating the Chromarods with silver nitrate (17,18). A method using Chromarods to effectively separate lipid classes based on differences in their fatty acid chain lengths has not been reported. Impregnating Chromarods with paraffins or silicones might give separations, but the lipids could not be detected using a flame ionization detector since the impregnating phase would also burn and produce ions. The partial separations within individual lipid classes on Chromarods reported here, based on differences in chain length and unsaturation of the fatty acid moieties, caused a reduction in resolution, particularly with biological lipids known to contain wide fatty acid profiles.

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COMMUNICATIONS

Effect of Dietary *trans* Fatty Acids on Microsomal Enzymes and Membranes

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ABSTRACT

Three groups of rats were maintained on diets containing different proportions of *trans* fatty acids (0, 18.3 or 36.6% of the total fatty acids) for eight weeks. No differences in body weight were observed among the three groups, but the fat cell size, determined in epididymal fat, differed significantly between the controls and the rats fed diets containing *trans* fatty acids. The supernatant obtained by centrifuging homogenates of liver from the rats at 9000 × g (S-9 fraction) was used as an activator in a bacterial test for mutagenicity of 2-aminofluorene and aflatoxin B_1 using *Salmonella typhimurium* strains TA 98 and TA 100, respectively. The mutagenicities of 2-aminofluorene in strain TA 98 and of aflatoxin B_1 in strain TA 100 were significantly lower with the liver S-9 fraction from rats fed a diet containing 36.6% *trans* fatty acids than with the liver S-9 fraction from rats fed a control diet with no *trans* fatty acids.
Lipids 20:620-624, 1985.

INTRODUCTION

The naturally-occurring unsaturated fatty acids are mostly of the *cis* configuration. Fatty acids in the *trans* form are not produced by animals or higher plants. But bacteria in the rumen can synthesize them, and they are also formed by hydrogenation of plant oils during the manufacture of margarines. Many margarines contain as much as 30 to 50% of *trans* fatty acids (1,2). Dietary *trans* fatty acids have been reported to be completely absorbed from the gastrointestinal tract in the rat (3-5) and to intensify essential fatty acid deficiency in this species (6,7). The distribution and levels of *trans* fatty acids in rat tissues have been reported to be influenced by the level in the diet (3). McConnell and Sinclair found that if rats were fed *trans* fatty acids their phospholipids contained as much as 30% of such fatty acids (8).

It is known that the fatty acid composition of phospholipids has an important influence on the physical properties of biological membranes (9). Awasthi et al. reported effects of phospholipids on different mitochondrial enzymes (10). Further, Ingelman-Sundberg et al. have demonstrated the effect of phospholipid vesicles on the catalytic properties of liver microsomal cytochrome P-450 (11). In the rat, feeding a low fat diet has been reported to result in a markedly lower activity of benzpyrene hydroxylase (a microsomal enzyme) (12).

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Membrane-bound enzymes in the liver microsomal fractions are involved in the mutagenic activation of aflatoxin B_1 and 2-aminofluorene (13-16).

The present study was carried out to investigate whether dietary *trans* fatty acids could influence the membrane-bound enzymes which are important for the metabolism of 2-aminofluorene and aflatoxin B_1.

MATERIALS AND METHODS

Chemicals

Aflatoxin B_1 and 2-aminofluorene were purchased from the Sigma Chemical Company. All solvents and reagents were of analytical grade.

Animals and Diets

Four-week-old male Sprague-Dawley rats were obtained from Anticimex. After 1 week of acclimatization, they were divided randomly into three groups of 10 rats each. The rats were housed in individual cages and were fed for eight weeks ad libitum with a basic fat-free diet to which 10% w/w fat had been added. The fat-free diet consisted of casein (19%), starch (63%), a vitamin mixture (1%), a salt mixture (4%) and cellulose powder (3%). The vitamin and salt mixtures in the diet were chosen according to Alling et al. (17). The fat mixtures for the different diets were composed of varied amounts of different fats and oils in order to keep the fatty acid composition as similar as possible, except for *trans* fatty acids. The fat and fatty acid compositions of the three diets are shown

in Table 1. The rats were not treated with any inducer of the microsomal enzyme system.

Fatty Acid Composition

Total fat from the diets and epididymal adipose tissue were extracted according to Folch et al. (18) after storage at -90 C. Phospholipids of adipose tissue total fat were separated on TLC (19). Methyl esters (20) were separated on GLC as follows: Dietary fat and adipose tissue total fat: 50 m Silar 10 C glass capillary column (21), 195 C isothermally. Split injection (split ratio 1:50). Phospholipids: 25 m OV-351 fused silica capillary column, temperature program 70 C 1 min, 25 C/min to 210 C. Split-less injection. Phospholipid *trans* fatty acids: 50 m Silar 10 C, temperature program 70 C 2 min, 25 C/min to 195 C. Split-less injection. A Varian GC equipped with a Hewlett-Packard capillary injection system 18835B and a Hewlett-Packard reporting integrator 3388A were used. The GC system was checked with Nu-Chek Prep, Elysian, Minnesota, USA, GLC reference mixtures. Total *trans* fatty acid contents of the diets were measured with an IR-technique (22).

TABLE 1

Fat and Fatty Acid Composition of the Diets

	Diet		
	Control	I	II
Fat[a]			
Olive oil	61	30	–
Grapeseed oil	3	8	11
Linseed oil	3	4	4
P.H. soybean oil[b]	–	42	85
Lard	33	16	–
Fatty acids[c]			
16:0	15.9	13.0	10.7
18:0	9.4	12.8	16.4
Σ saturated	26.4	26.7	27.8
trans-18:1	0.4	16.1	32.6
cis-18:1	59.8	43.3	25.6
Σ monoenes	61.7	60.2	58.2
trans-18:2[d]	tr[e]	tr	1.5
18:2 n-6	10.0	10.6	9.8
18:3 n-3	2.0	2.5	2.6
Σ polyenes	12.0	13.1	13.9
Total *trans*[a,f]	tr	18.3	36.6
P/S-ratio[g]	0.45	0.49	0.45

[a]Per cent of total fat.
[b]Partially hydrogenated soybean oil.
[c]Per cent of total fatty acids.
[d]*trans,trans,+trans,cis* + *cis,trans*-18:2.
[e]Trace amounts, <0.5 per cent.
[f]Analyzed with IR technique.
[g]$\frac{\text{18:2 n-6 + 18:3 n-3}}{\Sigma \text{ saturated}}$

Microsomal Fraction

The animals were decapitated and the livers removed under sterile conditions, weighed, immediately frozen and stored at -90 C for a few days. After thawing, the livers were homogenized in 3 vol of 0.15 M KCl at 1000 rev/min in a Potter-Elvehjelm homogenizer and centrifuged at 9000 x g for 10 min. The temperature was maintained at 4 C throughout. After centrifugation, the pellets were discarded and the supernatants (S-9 fractions) from 10 livers from each group were pooled and aliquots frozen at -90 C. The final preparation of the metabolizing system (the S9 mix) was carried out by the method of Ames et al. (23).

Mutagenesis Assay

Salmonella typhimurium strains TA 98 and TA 100 were used according to the procedure described by Ames et al. (23). 50 μl of liver homogenate (1.5 mg protein) was added to each plate. The test compounds (2-aminofluorene and aflatoxin B_1) were dissolved in dimethylsulfoxide and added to plates in 0.1 ml portions containing either 1, 2, 3 or 4 μg of 2-aminofluorene or 10, 20, 30 or 40 mg of aflatoxin B_1.

Fat Cell Size

The epididymal fat pads were removed by cutting close to the epididymis. The adipose tissue was then stored at -90 C until the cell size was determined by a microscopic method according to Sjöström et al. (24).

Protein Determination

The protein concentration in the liver homogenates was determined by the Lowry method (25), using bovine serum albumin as standard.

RESULTS

There were no differences in body weight among the three groups of rats fed diets with different amounts of *trans* fatty acids. However, the fat cell size, determined in the epididymal fat, was significantly smaller in the control group than in the groups fed diets containing 18.3% ($P < 0.01$) or 36.6% ($p < 0.005$) *trans* fatty acids (Table 2). (Statistical evaluation of differences by Student's t-test [26]).

Trans fatty acids are incorporated into total fat and phospholipids of the adipose tissue (Table 3). The incorporation into the total fat is approximately proportional to the *trans* fatty acid content of the diets. In the phospholipids the incorporation is the same in both groups. There is a tendency to lowered 20:4 n-6/18:2

n-6 ratios in phospholipids of the experimental groups, indicating effects on essential fatty acid metabolism. The differences are not significant, though.

Both 2-aminofluorene and aflatoxin B_1 were mutagenic toward *Salmonella typhimurium* (TA 98 and TA 100) in the presence of a rat liver S-9 fraction, which was used as a metabolizing system. The number of his $^+$ revertants increased with increasing concentrations of 2-aminofluorene and aflatoxin B_1. The mutagenicity of 2-aminofluorene in *Salmonella typhimurium* TA 98 was significantly lower with the liver S-9 fraction from rats fed a diet containing 36.6% *trans* fatty acids than with the liver S-9 fraction from rats fed a control diet with no *trans* fatty acids. However, there were no differences in the numbers of his $^+$ revertants obtained with the liver S-9 fraction between rats fed a diet containing 18.3% *trans* fatty acids and the rats on the control diet (Fig. 1). Aflatoxin B_1 also was used as a test substance for the liver S-9 fractions. The mutagenicity of aflatoxin B_1 in *S. typhimurium* TA 100 was lower with the liver S-9 fraction from rats fed a diet containing 36.6% *trans* fatty acids than with the liver S-9 fraction from the control rats. A small difference in the mutagenicity of aflatoxin was seen between the control group and the group fed a diet containing 18.3% *trans* fatty acids (Fig. 2).

TABLE 2

Body Weight and Fat Cell Size in Adipose Tissue of Rats Fed Different Amounts of *trans* Fatty Acids

Diets	Body weight (g)	Fat cell size (μm)
Control diet[a]	373 ± 26 ---	71 ± 6 ---
Diet I	381 ± 16 ns	80 ± 5 P<0.01
Diet II	384 ± 16 ns	85 ± 5 P<0.005

Mean value ± S.D. are given, n=10.

Student's t-test used for statistical evaluation. The Diet I and Diet II groups are compared with the control group. ns = Not significant.

[a]Total *trans* fatty acid content in the diets: <0.5% (control), 18.3% (I) and 36.6% (II).

DISCUSSION

Growth-suppressing effects of *trans* fatty acids in weanling rats have been demonstrated: these acids have an antagonistic effect on the growth response of essential fatty acid-deficient rats to linoleic acid (27). In the present study the *trans* fatty acid diets contained essential fatty acids and did not appear to inhibit growth. However, the fat cell size of the rats on diets containing *trans* fatty acids was significantly larger than that of the control group, which one would not have expected when the body weights were the same. This phenomenon may be the same as the reported swelling of mitochondria due to the significant incorporation of *trans* fatty acids into phospholipids, which are important constituents of biological membranes (28, 29). Furthermore, it has been demonstrated that *cis* and *trans* fatty acids impart different physical properties to phospholipids (9).

The present investigation also demonstrated inhibition of the mutagenicity of the test substances by the microsomal fractions of the

TABLE 3

Fatty Acid Composition of Total Fat and Phospholipids from Adipose Tissue of Rats Fed Different Amounts of *trans* Fatty Acids

Fatty acid (% of total fatty acids)	Total fat			Phospholipids		
	Control diet[a]	Diet I	Diet II	Control diet	Diet I	Diet II
16:0	21.7 ± 0.58	19.9 ± 0.30	19.9 ± 0.86	16.9 ± 2.29	14.0 ± 0.78	13.2 ± 0.48
18:0	4.2 ± 0.13	5.2 ± 0.11	5.5 ± 0.32	19.4 ± 1.11	16.9 ± 1.53	15.9 ± 1.18
trans-18:1	0.3 ± 0	10.0 ± 0.19[b]	17.1 ± 0.54[b]	2.2 ± 1.28	7.9 ± 1.04[b]	8.7 ± 0.60[b]
cis-18:1	59.2 ± 0.58	45.8 ± 0.59	35.4 ± 0.20	18.2 ± 1.23	16.5 ± 1.18	16.1 ± 1.31
18:2 n-6	7.6 ± 0.31	9.8 ± 0.22	10.3 ± 0.36	13.8 ± 0.88	16.5 ± 2.57[d]	16.9 ± 1.95[c]
20:4 n-6	–	–	–	11.8 ± 2.70	9.6 ± 0.36[d]	9.2 ± 1.46[d]
20:4 n-6 / 18:2 n-6				0.87 ± 0.240	0.60 ± 0.095[d]	0.55 ± 0.051[d]

Mean values ± S.D. are given, n=5. Student's t-test used for statistical evaluation. The Diet I and Diet II groups are compared with the control group.

[a]Total *trans* fatty acid content in the diet: <0.5% (control), 18.3% (I) and 36.6% (II).

[b]$P < 0.001$.

[c]$P < 0.05$.

[d]Not significant.

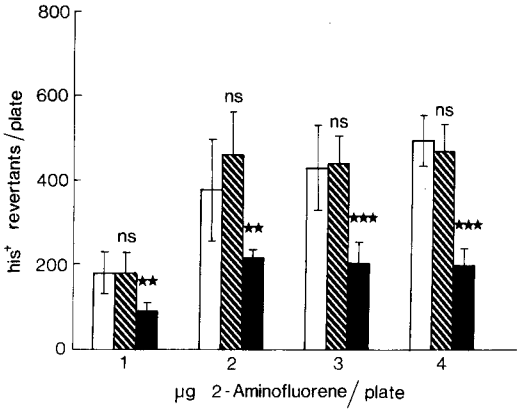

FIG. 1. The mutagenic response of TA 98 to various doses of 2-aminofluorene activated by 30 μl of S-9 fraction prepared from the livers of rats fed different diets: control diet = open columns, diet I = cross-hatched columns, diet II = filled columns. Each column represents the mean value of two different experiments with three plates/concentration. Mean values ± S.D. are shown. Student's t-test was used for statistical evaluation. ns = Not significant, * $P<0.05$, ** $P<0.005$ and *** $P<0.0005$.

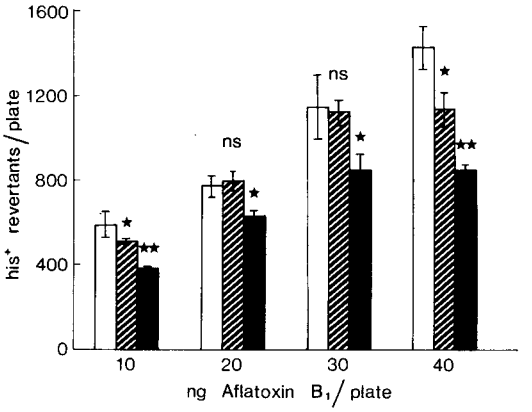

FIG. 2. The mutagenic response of TA 100 to various doses of aflatoxin B_1 activated by 50 μl of S-9 fraction prepared from the livers of rats fed different diets: control diet = open columns, diet I = cross-hatched columns, diet II = filled columns. Each column represents the mean value of two different experiments with three plates/concentration. Mean value ± S.D. are shown. Student's t-test was used for statistical evaluation. ns = Not significant, * $P<0.05$, ** $P<0.005$.

trans fatty acid-fed groups. This phenomenon indicates that the membrane-bound enzymes involved in the metabolism of the active metabolites of the test substances are influenced by dietary *trans* fatty acids. It is not possible to say if this inhibition of mutagenicity of aflatoxin B_1 and 2-aminofluorene is due to increased formation of the active metabolites or to increased degradation. Further studies are needed to determine if a high concentration of dietary *trans* fatty acids influences the metabolism of xenobiotics and if the influence of *trans* fatty acids on the enzymes is due to effects on specific enzymes or to a more general effect on membranes.

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Lipid Peroxidation and Oxidation of Several Compounds by H_2O_2 Activated Metmyoglobin

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ABSTRACT

Activated metmyoglobin (MetMb) by H_2O_2 initiates oxidation of microsomal unsaturated fatty acids, β-carotene and methional but not formate. Lipid peroxidation by activated MetMb was not inhibited by catalase. The activated species which initiates lipid peroxidation appears to be a porphyrin cation radical, $P^{+}Fe^{IV}=O$, and not a hydroxyl radical.
Lipids 20:625-628, 1985.

INTRODUCTION

Peroxidation of polyunsaturated fatty acids (PUFA) is a naturally occurring free radical chain reaction that has been implicated as a mechanism of tissue damage (1). For many years, one of the most interesting subjects was the reaction(s) leading to the production of such catalysts which initiate PUFA peroxidation. Various free radicals, such as hydroxyl radicals (2,3), $\cdot CCl_3$ (4), $NO_2^{\cdot}$ (5), $Cl^{\cdot}$, $I^{\cdot}$ (6) and ferry ion (7), were implicated in the initiation of lipid peroxidation.

Knowledge of the exact nature of the predominant forms of iron catalysts in biological tissues which are involved in the toxicity of lipid peroxidation in situ is lacking (8).

Hemoglobins and myoglobins play essential roles in maintaining aerobic metabolism. The autoxidation of oxymyoglobin and oxyhemoglobin leads to the formation of MetMb or MetHb and $O_2^{\cdot}$, which dismutate to H_2O_2 (9,10).

Metmyoglobin has the same prosthetic group as peroxidase and catalase, but its interaction with hydrogen peroxide is at a lower rate (11). The interaction of MetMb with H_2O_2 has been reported to produce a free radical (12), as subsequently confirmed by others (13,14). The MetMb radical also was formed as a result of reaction with organic hydroperoxides (15).

Shiga and Imaizumi (14) proposed that the hemeprotein radical of the MetHb-H_2O_2 system possessed chemical reactivity similar to that of the intermediate Compound I of horseradish peroxidase.

Compound I, based on recent NMR, ESR and electron nuclear double resonance, is best described as a porphyrin cation radical, $P^{+}Fe^{IV}=O$ (6).

Recently, seven hemeproteins were tested for their ability to catalyze a set of oxidative reactions (17).

The present study was conducted especially to demonstrate the ability of MetMb activated by H_2O_2 to oxidize microsomal lipids, and several other compounds.

MATERIALS AND METHODS

Formate-Na(^{14}C) with specific activity of 4-4-5 mCi/mmole was obtained from New England Nuclear, Boston, Massachusetts. All the reagents were of the highest purity commercially available.

Isolation of the microsomal fraction from dark turkey muscle tissue was done by a procedure described by Apgar and Hultin (18).

Microsomes for lipid peroxidation assay were incubated in a shaking water bath at 37 C in air. The reaction mixture contained 1 mg microsomal proteins per ml, and MetMb-H_2O_2 complex, 0.12 M KCl, 5 mM histidine buffer, pH 7.0. The reactions were initiated by the addition of MetMb-H_2O_2. Thiobarbituric acid reactive substances were determined by the procedure of Bidlack et al. (19). The results are reported as nmole malondialdehyde (MDA) per mg protein, using a molar extinction coefficient of $E_{532} = 1.56\ 10^5\ M^{-1}\ cm^{-1}$. The accumulation of conjugated dienes produced during lipid peroxidation was monitored by the increase in A_{234} nm (20). β-Carotene destruction, at pH 7.0 and 37 C, was determined by a method described previously (6).

The oxidation of ascorbic acid was determined spectrophotometrically using the decrease of the compound in absorbance at 265 nm, with time, by a DB Varian spectrophotometer DMS 90 connected to a recorder.

Methional (1 mM, in phosphate buffer pH 7.0) degradation to ethylene was conducted in a glass tube closed with a serum cap and incubated in a shaking water bath at 25 C. The ethylene produced in the reaction was identified with the use of a 6-ft Porapak Q column and

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a flame ionization detector in a Packard gas chromatograph. The amount of ethylene was calculated from a standard sample.

The oxidation of sodium formate (^{14}C) to $^{14}CO_2$ was determined using a method developed by May and Haen (21). Catalase or activated MetMb and format-Na (1 mM) containing 0.5 μCi (^{14}C) in buffer acetate 0.05 M, were incubated for 10 min at 37 C. The reaction was stopped by the addition of 0.5 ml acetic acid 1N to 4.0 ml reaction samples. $^{14}CO_2$ collected by KOH-soaked filter paper was transferred to a polyethylene scintillation vial, and 10 ml of Aqualume was added. The radioactivity was counted with 70% efficiency with a Kontron Liquid Scintillation Counter MR 300.

Protein determinations were conducted by the modified Lowry procedure (22), using BSA as standard.

The results are the mean of triplicates and, in the figures, each error bar (I) denotes the standard deviation.

RESULTS AND DISCUSSION

The nature of the reactive heme-oxygen species complexes differs depending on their oxidation states (23). Keilin and Hartree (1) showed that MetHb and MetMb in the presence of H_2O_2 form a complex which absorbs near 545 nm. Spectrophotometric determination of this compound in our model system showed a maximum concentration after 3 min of incubation at 25 C (Fig. 1). The specific spectra of activated MetMb changed rapidly with the addition of ascorbic acid. Simultaneously, ascorbic acid was oxidized (results not shown).

Of great interest is the finding that activated MetMb could initiate membranal lipid peroxidation. Lipid peroxidation was determined by accumulation of malondialdehyde (MDA) and conjugated dienes. The results from both methods show a significant increase in oxidized lipids. Lipid peroxidation by the activated MetMb was not inhibited by catalase (Table 1). We found that MetMb also could bleach β-carotene (Fig. 2), and this could be inhibited by ascorbic acids (results not shown).

The peroxidation of the unsaturated fatty acids and the destruction of β-carotene in the presence of activated MetMb indicates a possible attack on one of the double bonds, or on an allylic hydrogen and the production of a hydroperoxide. The abstraction of an electron from this position changes the specific absorption of β-carotene (6).

Our work shows that activated MetMb also has the ability to produce ethylene from methional. The conversion of methional to ethylene

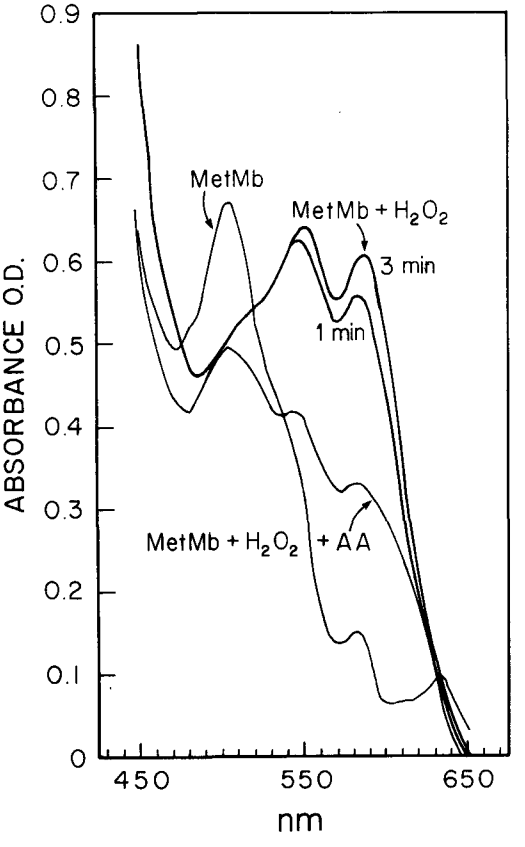

FIG. 1. Spectra changes of metmyoglobin (30 μM) incubated in the presence of H_2O_2 (30 μM) and after the addition of ascorbic acid (100 μM).

was dependent on the amount of H_2O_2 and gave an excellent yield of 70-80% (Fig. 3). Using the same technique, Pryor and Tang (24) reported yields of 42% and 17% for RO· (alkoxyl) and ·OH (hydroxyl) radicals, respectively.

The generation of ethylene from methional originally was proposed as an assay for hydroxyl radicals (25). However, Pryor and Tang (24) showed that a variety of organic radicals lead to the production of ethylene from methional.

Of all the molecules tested by us, only sodium formate was stable and not decomposed in the presence of activated MetMb. Formate (^{14}C) oxidation to CO_2 (^{14}C) by the peroxidative activity of catalase in the presence of low H_2O_2 concentration was determined as a positive control treatment (Fig. 4). As is well known, formate could be oxidized to CO_2 by catalase compound I (26) or by hydroxyl radicals (27). The redox potential of hydroxyl radical at pH 7.0 ($\cdot OH/H_2O$) is about +2.3 V (28), high enough to oxidize almost all bio-molecules;

TABLE 1

Microsomal Lipid Peroxidation Initiated by Activated MetMb[a]

Treatments	Malondialdehyde nmole/mg protein/30 min	Conjugated dienes A 233 nm/30 min
Microsomes alone	0	0.16
Microsomes + H_2O_2[b]	0	0.15
Microsomes + MetMb[b]	5.4 ± 2.7	0.16
Microsomes + MetMb/H_2O_2[c]	35.0 ± 2.4	0.31
Microsomes + MetMb + H_2O_2	32.4 ± 3.2	0.30
Microsomes + catalase + MetMb/H_2O_2	6.5 ± 3.0	0.15
Microsomes + catalase + MetMb + H_2O_2	32.0 ± 1.8	0.28

[a]MetMb + H_2O_2 the same conc. incubated for 3 min before addition.

[b]H_2O_2 or MetMb at a conc. of 30 μM each.

[c]MetMb/H_2O_2 the same conc. not incubated for 3 min.

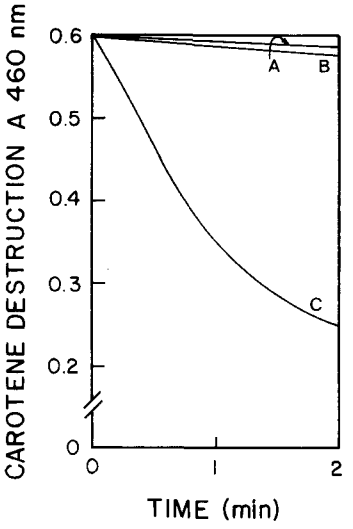

FIG. 2. Carotene oxidation by activated metmyoglobin. A, metmyoglobin (30 μM); B, H_2O_2 (30 μM); C, metmyoglobin (30 μM) and H_2O_2 (30 μM) incubated for 3 min before addition.

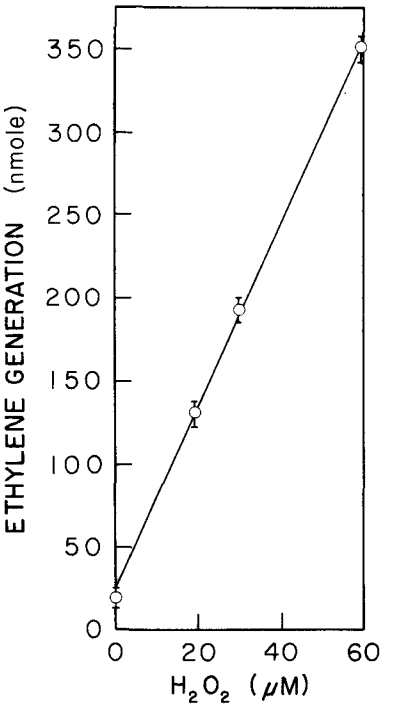

FIG. 3. The effect of hydrogen peroxide concentration on the generation of ethylene from methional by activated metmyoglobin (30 μM). Reaction mixture contained buffer phosphate (1 m), EDTA (1 mM) at pH 7.0 and 25 C. Control H_2O_2 (60 μM) without the addition of metmyoglobin = 34 nmole ethylene.

however, activated MetMb was found to have a redox potential of only +1.4 V (14). This potential of activated MetMb was high enough to oxidize molecules such as polyunsaturated fatty acids (PUFA), β-carotene, methional, ascorbic acid and phenol red (data not shown), but not enough to oxidize formate.

Our results show that activated MetMb, probably a porphyrin cation radical $P^{\pm}$ Fe^{IV}=O, initiates membranal lipid peroxidation in a model system. Similar results were obtained using an activated methemoglobin (29).

More recently, it was shown that activated neutrophils, which produce O_2^- and H_2O_2 extracellularly by contact with intact erythrocytes, mediate methemoglobin formation and lysis. It was proposed that methemoglobin reacted with H_2O_2 within the erythrocyte to form a powerful oxidant which attacked the membrane from within (3o).

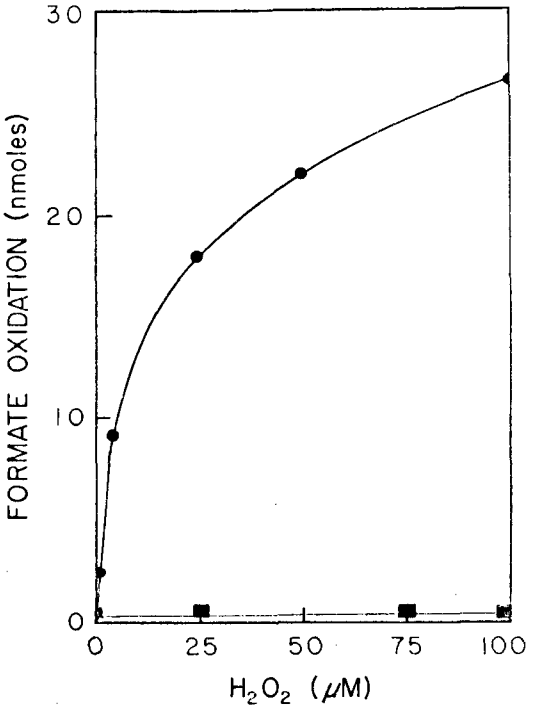

FIG. 4. Formate oxidation by catalase and metmyoglobin affected by H_2O_2 concentration. •, catalase; ▪, metmyoglobin. Reaction mixture contained catalase (425 U) or metmyoglobin (30 μM), formate-Na 1 mM contained 0.5 μCi (^{14}C) in buffer acetate (0.05 M) pH 6.5, for 10 min at 37 C.

Our results indeed demonstrate the powerful oxidant ability of activated MetMb and MetHb to attack several molecules and to initiate membranal lipid peroxidation.

ACKNOWLEDGMENT

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Unusual Predominance of Even-Carbon Hydrocarbons in an Antarctic Food Chain[1]

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ABSTRACT

A comparative study of lipids was conducted in *Euphausia crystallorophias, Pleuragramma antarcticum* and *Dissostichus mawsoni,* representing three trophic levels of an Antarctic food chain. Very large concentrations of triglycerides were found in *P. antarcticum* and *D. mawsoni,* but the nature of the free fatty acids and triglyceride fatty acid distributions of all three species was unremarkable. The larger amounts of free fatty acids obtained in *Euphausia crystallorophias,* when compared to a previous analysis, may have been due to enzymatic hydrolyses of lipid esters during cold storage. In contrast, the hydrocarbon distributions in the three organisms were found to contain an unusual predominance of even-carbon *n*-alkane constituents. A different biochemical pathway to hydrocarbons may be operating in members of this food chain that is so well adapted to the low temperatures of Antarctic waters. *Lipids* 20:629-633, 1985.

INTRODUCTION

A unique group of teleost fishes of the suborder *Notothenioidei* inhabits the cold waters (0 to -1.9 C) of the Southern Ocean surrounding the Antarctic continent. Marine food chains in low-temperature Antarctic waters are often less complex than their temperate water counterparts (1). We therefore decided to conduct a comparative study of the lipids in two of these nototheniid fishes and one crustacean, representing three trophic levels of an Antarctic food chain in the Ross Sea-McMurdo Sound region. The organisms were: a krill, *Euphausia crystallorophias* (EC); Antarctic herring, *Pleuragramma antarcticum* (PA), and Antarctic cod, *Dissostichus mawsoni* (DM).

The large (up to 2 m) fish, DM, is a semipelagic species which lives in open water and beneath the ice and feeds throughout the water column south of the Antarctic convergence. The primary food of DM, from analysis of stomach contents, are PA, squid, mysids and the fish *Trematomous borchgrevinki.* PA is a circumpolar pelagic species abundant in the coastal Antarctic waters and feeds primarily on herbivorous-omnivorous plankters. The stomach contents of the PA specimens used in this work were composed primarily of EC, a fact previously noted by DeWitt (2). The main component of Antarctic krill, *Euphausia superba* (ES), are selcom caught this far south in the Ross Sea (3) and were not found in the PA stomach contents. EC is found primarily in the Ross Sea adjacent to the ice shelf, in McMurdo Sound, and along the coast of Victoria land (4).

While the lipid content of EC was generally consistent with previous non-hydrocarbon lipid analyses of krill species, primarily ES but including one study of EC (5-12), the hydrocarbon distribution was found to contain a highly unusual predominance of even-carbon *n*-alkane constituents. This even-chain *n*-alkane dominance also was observed in the two nototheniid fishes, PA and DM. Lipid analysis data recently brought to our attention on the related crustacean ES, PA larvae and a number of other Antarctic midwater organisms confirmed the predominance of even-carbon hydrocarbons (Reinhardt, S.B., and Van Vleet, E.S., submitted for publication) in members of this unique ecosystem and prompted this publication of unusual hydrocarbon data.

EXPERIMENTAL

EC and PA were collected on Feb. 24, 1972 on cruise 51 of the Eltanin at Station 18 in the Ross Sea (77°0.56'S, 172°44.4'E) with a 3 m Isaacs-Kidd midwater trawl for 2 hr from the surface to 200 m (bottom depth, 400 m). Specimens were transferred immediately to carbon-free glass jars with teflon-lined caps and frozen at -20 C. Precautions were taken to prevent the cod ends of the nets from touching the ship's deck or allowing the specimens to be unduly exposed to the ship's atmosphere (fuel vapors,

[1] Taken in part from a progress report prepared for the Energy Research and Development Agency (ERDA): Williams, P.M., and Nachman, R.J. (1975), University of California Institute of Marine Resources, Research of Marine Food Chain Progress Report, UCSD 10P20-202, 07/74 to 06/75, p. 151-153, prepared for ERDA, Div. of Biomed. and Environ. Res., Contract AT(11-1) GEN 10, P.A. 20.

[2] Work carried out at the Institute of Marine Resources, Scripps Institution of Oceanography, University of California at San Diego, La Jolla, CA 92093.

stack smoke, etc.). DM was caught near the bottom under the annual ice, 3 mi southwest of McMurdo Station, on Dec. 3, 1974. This 100-lb specimen of 1.5 m in length was caught on vertical set-line at 450 m using the fish *T. borchgrevinki* as bait. The fish was bled and frozen at -20 C within 1 hr. Analysis of the lipids of these organisms was carried out in late 1974 and early 1975.

After thawing and maceration, EC (whole organism), PA (white muscle) and DM (white muscle) were extracted with $CHCl_3$:CH_3OH (2:1) with stirring under N_2 at room temperature (RT) for 6 to 7 hr (13). Mixtures were filtered through celite, solvents stripped in vacuo at RT and the oil-water emulsions lyophilized. Oily residues then were extracted with hexane under N_2 and the hexane removed in vacuo at RT. The residual oil was taken up in hexane, chromatographed on activated silica gel (Davison Chemical, 60/100 mesh) and eluted with various proportions of petroleum ether, benzene-petroleum ether, and benzene-ethyl ether mixtures (14). Individual lipid classes in the eluates were identified by TLC, IR and NMR. Hydrocarbons were analyzed by injection into a Varian Aerograph Series 1400 gas chromatograph equipped with a 6 m x 2 mm inside diameter glass column packed with 3% OV-101 on Gaschrom Q, 100/200 mesh. Analyses were run isothermally at 180 C, and identification of hydrocarbons was effected by (i) co-chromatography with standard saturated *n*-alkanes (C_{11}-C_{22}) and monounsaturated *n*-alkanes (C_{10}-C_{22}) (Applied Sciences Laboratories), and (ii) GC-MS using a Hewlett Packard (HP) Model 5700 gas chromatograph coupled to a HP Model 5930A mass spectrometer with HP Model 2100S systems computer. Pristane was identified by GC-MS and by its retention time as compared to an appropriate literature value (15). None of the GC traces showed the typical "envelope" characteristics of petroleum hydrocarbons. Free fatty acids (FFA) and triglyceride fatty acids (TFA) were identified as their methyl esters after transesterification with BF_3-CH_3OH (16) by GC-MS and co-chromatography with standard saturated, monounsaturated and polyunsaturated methyl esters (Applied Science Laboratories). Wax esters were chromatographed on the OV-101 column operated isothermally at 300 C and identified by co-chromatography with spermacetti wax (predominantly 28:0, 30:0, 32:0, 34:0 and 36:0) and copepod *Calamus plumchrus* wax esters (primarily 30:1, 32:1, 34:1, 36:1 and 38:1) (17).

TABLE 1

Distribution of Lipid Classes and the Total Lipids of EC, PA and DM (wt %)[a]

Lipid class	EC	PA	DM
Hydrocarbons	1.2	0.7	0.2
Free fatty acids	25.8	5.4	2.4
Triglycerides	30.1	74.9	60.1
Wax esters	9.2	0.7	0.6
Sterol esters	3.5	2.5	0.9
Complex lipids	30.2	15.8	35.8
Total lipid (% of dry wt)	12	39	23
Dry wt (% of wet wt)	20	20	42

[a]EC, *Euphausia crystallorophias*; PA, *Pleuragramma antarcticum*; DM, *Dissostichus mawsoni*.

RESULTS AND DISCUSSION

Table 1 gives the total lipid content and distribution of lipid classes in EC, PA and DM. The total lipid content of EC, 12% of its dry wt, is not significantly higher than is normally found in pelagic euphausids from more temperate waters whose lipid contents are 7 to 12% (mean of 4 species: 10%) (18). The high concentration of lipid in PA and DM white muscle is composed primarily of triglycerides with relatively low concentrations of wax esters. A similar lipid distribution was found by Patton (20) in a specimen of *T. borchgrevinki* caught directly beneath the ice in McMurdo Sound, with triglyceride representing 80% of the lipids (total lipid content: 30% dry wt). While the high concentrations of triglycerides in PA and DM represent potential energy stores, they also may function as buoyancy agents (21) (the density of triglycerides is 0.92 at 21 C [24]). Neither PA nor DM possess swim bladders; thus, the triglycerides may be responsible for the virtual neutral buoyancy observed (22,23) in both PA and DM and necessary for vertical migration. The wax esters of EC and PA are monounsaturated and constituted primarily of 18:1 and 16:1 fatty acids coupled with 14:0 and 16:0 fatty alcohols. The distribution and composition of wax esters in EC (Table 3) is essentially in agreement with a previous analysis (5) of the waxes in EC. The free fatty acid (FFA) and triglyceride fatty acid (TFA) constituents (Table 2) in EC are generally consistent with previous lipid analyses of a variety of *Euphausia* species (5-12). However, our results for the lipid distribution in EC differ from Bottino's (12) mainly in the high amounts of free fatty acids isolated in this work. The high percentages of free fatty acids encountered in this work probably are due to the enzymatic hydrolyses of varying amounts of different lipid esters in the frozen sample of EC during storage and thawing (25). The fatty acid components

in both PA and DM are quite unremarkable (Table 2).

In contrast, with the exception of pristane and 21:1, all hydrocarbons isolated in significant amounts from EC, PA and DM had even chain lengths (Table 1 and Fig. 1). A predominance of even-carbon normal hydrocarbons is most uncommon in plants and animals of both marine and terrestrial origin (26-31). An even-carbon *n*-alkane preference has been reported previously in the fungus *Trichoderma viridae* and several species of the bacterial *Arthrobacter, Micrococcus* and *Mycobacterium* genera (27,31-34).

A few examples also have been reported among marine species. Two species, the Atlantic Sand Launce (*Ammodytes americanus*) and the Pacific Grayfish (*Squalus sukleyi*), have been reported to have a predominance of even-carbon *n*-alkanes (35). At the conclusion of a survey of saturated hydrocarbons in marine organisms, Mironov et al. have reported a number of species in which the dominant hydrocarbon has an

TABLE 2

Distribution of Hydrocarbons in EC, PA and DM (in wt %)[a]

Hydrocarbon	EC	PA	DM
14:0	3.9	0.6	25.8
16:0	27.3	18.7	52.7
16:1	–	0.5	2.4
18:0	8.5	1.8	10.9
18:1	35.2	11.5	8.2
19:0[b]	0.5	47.8	–
20:0	8.2	5.3	tr
20:2	3.2	6.1	–
21:1	13.2	7.7	–

[a]EC, *Euphausia crystallorophias*; PA, *Pleuragramma antarcticum*; DM, *Dissostichus mawsoni.*

[b]Pristane.

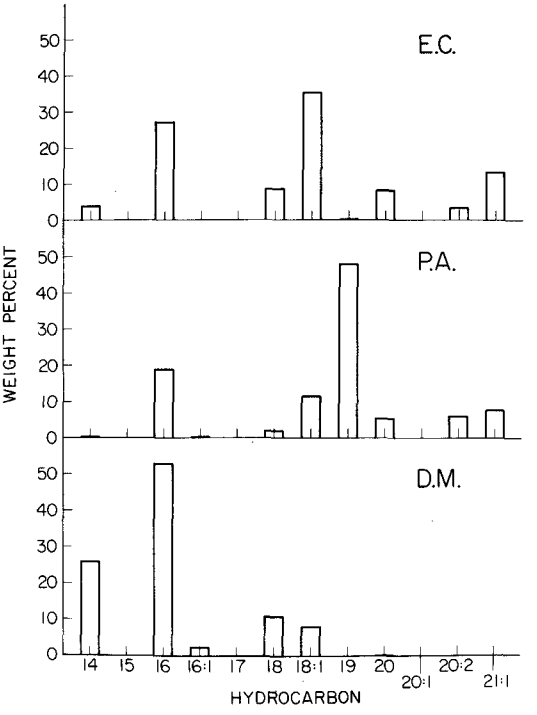

FIG. 1. The hydrocarbon distribution in *Euphausia crystallorophias* (EC), *Pleuragramma antarcticum* (PA) and *Dissostichus mawsoni* (DM).

TABLE 3

Distribution of Free Fatty Acids (FFA) and Triglyceride Fatty Acids (TFA) in EC, PA and DM (wt %)[a]

Fatty acid	EC		PA		DM	
	Free FFA	Triglyceride TFA	Free FFA	Triglyceride TFA	Free FFA	Triglyceride TFA
14:0	3.3	38.7	10.1	21.2	9.6	15.1
16:0	6.1	24.2	10.0	11.6	14.1	15.1
16:1	9.8	0.7	10.7	20.0	17.1	16.9
18:0	–	18.1	–	5.8	–	–
18:1	18.9	3.2	40.0	25.8	36.5	38.3
18:2	tr	–	tr	–	tr	–
19:0	0.5	–	–	–	0.5	–
20:1	–	1.1	4.9	2.3	4.7	4.2
20:2	–	–	–	–	–	1.3
20:4	11.7	2.3	3.2	2.7	2.4	0.6
20:5	27.2	5.3	8.5	8.1	10.2	2.5
22:1	–	2.0	4.9	0.7	2.2	1.3
22:6	22.5	4.4	7.4	1.8	2.2	4.7
24:1	–	–	0.3	–	0.5	tr

[a]EC, *Euphausia crystallorophias*; PA, *Pleuragramma antarcticum*; DM, *Dissostichus mawsoni.*

even chain length, but the reference does not indicate the total chain length preference or the carbon preference index (CPI) for these organisms (36).

Finally, Clark et al. (37) have reported a slight even-carbon preference among C_{20}-C_{36} hydrocarbons (CPI_{20-36} = 0.9) for both mussels (*Mytilus edulis*) and oysters (*Ostrea lurida*) exposed to outboard motor effluent, but not among controls. In addition, an odd carbon preference was retained among C_{14}-C_{20} hydrocarbons (CPI_{14-20} = 1.3 and CPI_{14-20} = 5.6, respectively) for both species. Therefore, the strong even-carbon *n*-alkane preference, at the virtual exclusion of odd *n*-alkanes, noted in the Antarctic food chain is a highly unusual situation. Extraction of an additional portion of EC from Station 18 gave identical results. Blank determinations on the whole procedure (solvents, silicic acid, N_2 gas, etc.) were negative for even- or odd-numbered alkanes, indicating that the possibility of petroleum contamination was most unlikely.

The results from this Antarctic food chain are in agreement with recent data of Van Vleet (Reinhardt, S.B., and Van Vleet, E.S., submitted for publication) who has found even-carbon dominance among hydrocarbons of the related crustacean ES, PA larvae and a number of other Antarctic midwater organisms. Fatty acid distribution data were similar as well. The most considered pathways for *n*-alkane biosynthesis are the elongation-decarboxylation and condensation-reduction pathways and modifications thereof (27,31,38) involving fatty acid precursors. Thus, relatively high concentrations of odd-numbered rather than the observed even-numbered fatty acids (Tables 3,4) might be expected. This was not the case, and suggests that a different biosynthetic pathway may be operating in these predatory members or, alternatively, in the lower phytoplanktonic members of this food chain that is so well adapted to the low temperatures of the waters surrounding the Antarctic continent. One possibility might be that these hydrocarbons arise via the dehydration of fatty alcohols followed by reduction (Kolattukudy, personal communication). In any case, an investigation of the *n*-alkane biosynthetic pathway operating in these organisms would seem warranted.

TABLE 4

Distribution of Wax Esters in EC, PA and DM (in wt %)[a]

Wax ester	EC	PA	DM
26:1[b]	0.2	1.0	17.5
28:1[b]	1.9	7.3	82.5
30:1	11.2	22.2	—
31:1	0.4	12.7	tr
32:1	50.4	32.9	tr
34:1	34.6	19.7	tr
36:1	1.3	4.2	tr

[a]EC, *Euphausia crystallorophias*; PA, *Pleuragramma antarcticum*; DM, *Dissostichus mawsoni*.

[b]Not unequivocally identified as wax esters.

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Cardiac Lipid Changes in Rats Fed Oils Enriched in Saturates and Their Apparent Relationship to Focal Heart Lesions

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ABSTRACT

Weanling male Sprague-Dawley rats were fed diets containing 20% by weight corn, soybean or low erucic acid rapeseed oils or mixtures of the latter two with cocoa butter or triolein for 1, 2, 3 or 4 weeks. These diets previously had been fed to the same strain of rats for 16 weeks, and a reduction in the incidence of focal heart lesions had been observed with the addition of cocoa butter, but not triolein. The cardiac lipid classes and the fatty acid and alkenyl ethers of the cardiac phospholipids were analyzed to determine if changes could be attributed to the observed cardiopathological response, and at what time. Cardiac lipid classes changed during post-weaning development, but only triacylglycerol was diet-related. A number of fatty acid changes were observed in the cardiac phospholipids which reflected the relative concentration of saturates, monounsaturates, linoleic acid and linolenic acid in the diet, but only the changes in saturates and the C22 polyunsaturated fatty acids from the linolenic acid family appeared to be related to the incidence of focal heart lesions. Arachidonic acid and the total C22 polyunsaturated fatty acids remained fairly constant throughout the feeding trial. Cardiac diphosphatidylglycerol was least affected by dietary manipulation, while nervonic acid increased in cardiac sphingomyelin when small amounts of erucic acid were present in the diet. Fatty acid changes were essentially completed after one week on the experimental diets, whereas changes in the alkenyl ethers took two to three weeks.

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INTRODUCTION

Weanling male rats fed diets containing 20% by weight fats or oils for at least 16 weeks have been observed to develop focal heart lesions (1). The evidence suggests that the incidence of these lesions is associated with levels of specific dietary fatty acids: linolenic acid (18:3 n-3) (2-4) and erucic acid (22:1 n-9) (5) correlated positively, and saturated fatty acids (4,6) and linoleic acid (18:2 n-6) (3,4) correlated negatively.

In a recent study, the relationship between dietary fatty acids and focal heart lesions was examined (7,8). Male rats fed diets in which the relative concentration of saturated fatty acids in low erucic acid rapeseed (LEAR) oil and soybean oil was increased by about 10% with cocoa butter had a 40% reduction in the incidence of focal heart lesions. Substitution of a similar amount of triolein for cocoa butter had no effect on the incidence of lesions. The cardiac lipids of rats fed the same diets for 16 weeks were investigated (8) to determine if some specific qualitative and/or quantitative lipid changes might be related to the observed cardiopathological response. High levels of C22 polyunsaturated fatty acids (PUFA) and lower levels of saturates appear to be associated with this cardiopathological condition at 16 weeks.

The present study was undertaken to determine the time course of cardiac lipid changes and compositional changes in cardiac tissues of rats fed oils differing in their cardiopathological response which might reveal patterns which lead to focal heart lesions. A diet containing corn oil also was included to compare the effects of an oil low (0.9%) in 18:3 n-3 to LEAR and soybean oils, which both contain substantial (7-10%) amounts of 18:3 n-3. The fatty acid compositions of the major cardiac phospholipids were investigated, namely, choline phosphoglyceride (CPG), ethanolamine phosphoglyceride (EPG), diphosphatidylglycerol (DPG) and sphingomyelin (SP), to see if and when a characteristic pattern might emerge which could be associated with the observed cardiopathological response.

MATERIALS AND METHODS

A total of 204 male weanling Sprague-Dawley rats (Bio-Research, Montreal, Quebec), weighing 40 to 50 g, were ear notched for identification and distributed randomly among 8 dietary groups of 24. The remaining 12 rats served as control and were killed immediately by CO_2 asphyxiation. The rats were housed 2 per cage and fed the test diets for 1, 2, 3 or 4 weeks. At the end of each time interval, 6 rats per diet (from 3 cages) were killed. Water and feed were provided ad libitum. The room temperature was maintained at 22 ± 1 C, and the animals received

12 hr of light (0800 to 2000). Body weights were recorded every week.

The test diets were formulated to contain 20% oil or oil mixtures, as shown in Table 1. Details of the basal diet have been reported previously (7). The amount of feed consumed weekly by a pair of rats per cage was determined by feed weigh back. The hearts were removed, weighed individually, and then combined in pairs for immediate lipid extraction as described previously (9).

The cardiac lipid classes were quantitated using an Iatroscan as described previously (8). The neutral lipids including cholesterol were separated using 1,2-dichloroethane/chloroform/formic acid (98/2/0.1) as developing solvent (10). The phospholipids were separated (11), and their fatty acid and alk-1-enyl ethers were prepared (12) for analyses by gas liquid chromatography (GLC).

A Hewlett-Packard Model 5830A GLC was used, equipped with flame ionization detectors, a digital integrator and a Hewlett-Packard 7671A Automatic Sampler. The flexible fused silica capillary column (30 m × 0.25 mm i.d.), coated with Carbowax 20M (Chromatographic Specialties Ltd., Brockville, Ontario), was operated isothermally at 195 C. Other particulars: injector and detector temperatures 250 C each; helium carrier gas at 1.1 ml/min through column; split ratio of 5:1; nitrogen make-up gas at 29 ml/min; hydrogen 30 ml/min; air 240 ml/min; injection volume of 3 μl, and attenuation of 4.

Analyses of variance were applied to all measurements. In addition, the initial body weights were used as covariates in analyzing later body weights. Least significant differences (LSD) were calculated using error estimates for analyses of variance of the 1- 4-week data inclusive. The 16-week data from a previous publication (8) are presented where appropriate, but the data were not included in any of the analyses. The LSDs of the 16-week data were lower than those of the 1- 4-week data for all parameters considered, mainly because of the difference in the number of values comprising each mean. Therefore, it was decided to use the 1- 4-week LSD estimates for comparison. Significance tests were conducted at the 1% level; unless otherwise noted, differences with probability less than 1% are indicated as significant.

RESULTS AND DISCUSSION

Growth Performance

The body weights of the rats fed the eight experimental diets are shown in Table 2. There were no significant differences in growth among the three pure vegetable oils, corn, LEAR and

TABLE 1

Dietary Fats and Their Fatty Acid Composition

Dietary oils[a]	Corn	LEAR	LEAR + CB	LEAR + 18:1	Soybean	Soybean + CB	Soybean + 18:1	Soybean + 18:1 + LCM
				% by weight of the diet				
Corn oil	20	–	–	–	–	–	–	–
LEAR oil	–	20	16	16	–	–	–	–
Soybean oil	–	–	–	–	20	16	9.6	9.5
Cocoa butter (CB)	–	–	4	–	–	4	–	–
Triolein (18:1)	–	–	–	4	–	–	9.6	9.5
Linseed oil	–	–	–	–	–	–	0.8	0.8
Long chain monoenes (LCM)[b]	–	–	–	–	–	–	–	0.2
				% by weight of dietary fat				
Fatty acids[c]								
Total saturates	12.6	7.2	16.6	5.4	16.2	27.6	8.7	9.1
Total monoenes	24.5	60.2	57.6	68.7	25.1	27.4	56.3	56.7
18:2 n-6	61.1	22.0	17.8	17.9	51.9	40.1	28.3	27.5
18:3 n-3	0.9	10.3	7.9	7.8	6.7	4.9	6.7	6.7

[a]LEAR = low erucic acid rapeseed; CB, cocoa butter; 18:1, triolein; LCM, long chain monoenes (20:1, 22:1, 24:1).

[b]Long chain monoenes = 79% 20:1, 20% 22:1 and 1% 24:1; a distillate obtained from the methyl esters of a high erucic acid rapeseed oil.

[c]Fatty acid expressed as % by weight and based on analysis of dietary fats.

TABLE 2

Body Weights and Feed Efficiency of Male Rats Fed Experimental Diets from Weanling Age[a]

Dietary oils[b]	Body weights (g)					Feed efficiency (weight gain/feed consumed)				
	1 wk	2 wks	3 wks	4 wks	16 wks[c]	1 wk	2 wks	3 wks	4 wks	16 wks[d]
	n=24[e]	n=18	n=12	n=6	n=50	n=12	n=9	n=6	n=3	n=50
Corn	85	120	159	217	–[f]	0.307	0.265	0.249	0.243	–[f]
LEAR	85	128	171	224	416	0.348	0.305	0.277	0.268	0.221
LEAR + CB	85	126	167	223	466	0.343	0.308	0.276	0.268	0.244
LEAR + 18:1	83	125	165	213	427	0.344	0.301	0.269	0.260	0.224
Soybean	81	121	165	216	496	0.309	0.271	0.234	0.243	0.257
Soybean + CB	81	123	170	218	508	0.296	0.262	0.238	0.226	0.260
Soybean + 18:1	81	112	150	197	461	0.294	0.240	0.225	0.218	0.237
Soybean + 18:1 + LCM	77	110	148	188	464	0.285	0.240	0.227	0.240	0.244
Analysis of variance (df)[g]	Mean squares[h]									
Among all diets (7)	189	747*	931	1002	54841*	.0077*	.0069*	.0028*	.0020	–[i]
Among original oils (2)	158	394	432	129	–	.0063*	.0042*	.0029	.0002	
Oils vs added CB (1)	2	1	11	1	48578*	.0009	.0001	.0001	.0002	
Oils vs added 18:1 (1)	209	1595*	2630*	3142	7200	.0063*	.0082*	.0015	.0009	
Error	114	194	384	721	2112	.0012	.0008	.0005	.0004	

[a]Mean initial body weight of male rats was 46 g for the 1- to 4- week study (192 rats) and 55 g for the 16-week study (350 rats) (7,8).

[b]LEAR, low erucic acid rapeseed; CB, cocoa butter; 18:1, triolein; LCM, long chain monoenes (20:1, 22:1, 24:1).

[c]Data taken from reference 8.

[d]Data taken from reference 7.

[e]n=Number of observations.

[f]Diet not fed.

[g]Degrees of freedom (df) shown in brackets; df for error can be calculated as 8×(n-1).

[h](*) = P<0.1.

[i]The LSD at P<0.01 is 0.0124 (7).

soybean. The addition of saturates in the form of cocoa butter to LEAR and soybean oils apparently did not affect body weights within the first four weeks on the diets. These results are consistent with previous studies in which no differences in growth were observed at four weeks (13,14), but growth differences were evident at 8, 12 and 16 weeks on trial (14). In general, the addition of triolein to LEAR and soybean oils resulted in a reduction in growth, which was significant for both week 2 and 3 data. The differences remained at four weeks but were not significant, owing mainly to the smaller sample size involved in the comparison. The consistent reduction in growth, when rats were fed the soybean oil-triolein mixtures (1:1), probably was due to the lower levels of saturates in these diets (8.7 and 9.1%) compared to soybean oil (16.2%).

Among the three vegetable oils, LEAR oil gave the best feed efficiency throughout the first four weeks (Table 2). While the addition of saturates to the dietary oils did not affect the feed efficiency, the addition of large amounts of triolein (i.e., 1:1 mixtures with soybean oil) reduced feed efficiency during the four weeks. The LEAR and triolein differences were significant only for the first two weeks, but, as noted above, the lack of significance in the later weeks probably was due to the smaller sample size.

Cardiac Lipid Changes

The analysis of heart weights, cardiac lipid content and cardiac lipid composition (Table 3) showed significant diet differences only for cardiac triacylglyceride (TG). The level of cardiac TG increased by about 70% when weanling rats were placed on these 20% fat diets, and increased only slightly throughout the remaining test period (Table 3). There were some significant diet differences for TG. Rats fed the soybean oil-based diets had higher levels of cardiac TG (8.5 mg/g) than either the corn (6.8 mg/g) or LEAR oil (7.3 mg/g) based diets, but the addition of triolein or cocoa butter to the oils did not markedly alter the cardiac TG level. Nagai et al. (15) found that the level of cardiac

TABLE 3

Heart, Lipid and Cardiac Lipid Class Weights Pooled Over All Diets

Description[a]	Time on diet (weeks) 0 n=12[b]	1 n=48	2 n=48	3 n=48	4 n=48	16 n=42	Statistics[c] Diets	Time 1-4 weeks	LSD
Heart (mg)	205	414	569	666	883	1107	NS	S	
Lipids (mg/g)[d]	35.1[e]	33.2[f]	32.7[f]	34.8[f]	38.3[f]	36.9[g]	NS	S	
CE (mg/g)[d]	0.6[e]	0.2[f]	0.2[f]	0.2[f]	0.2[f]	0.3[g]	NS	S	0.3
TG	4.6	7.7	6.5	8.2	8.6	9.9	S	S	3.5
C	3.5	3.2	2.6	2.8	3.0	2.2	NS	S	0.9
DPG	3.7	3.1	3.1	3.4	4.1	2.7	NS	S	1.1
EPG + IPG	5.9	5.6	6.1	6.7	7.6	6.4	NS	S	1.8
SPG	0.9	0.6	1.2	1.0	0.8	0.9	NS	S	0.5
CPG	12.5	9.9	10.4	10.6	11.5	9.3	NS	S	2.9
SP	1.9	1.4	1.6	1.0	1.3	0.8	NS	S	0.7

[a]CE, cholesteryl ester; TG, triacylglycerol; C, cholesterol; DPG, diphosphatidylglycerol; EPG, ethanolamine phosphoglyceride; IPG, inositol phosphoglyceride; SPG, serine phosphoglyceride; CPG, choline phosphoglyceride; SP, sphingomyelin.

[b]n = Number of rats per time period.

[c]LSD = least significant difference obtained from pooler error estimates of analysis of variance from the 1- to 4-week data. NS = not significant. S = significant ($P<0.01$).

[d]Weight is given in mg/g wet heart tissue.

[e]Values represent mean of 6 pairs of hearts from weanling male rats.

[f]Values represent mean of 3 pairs of hearts from each of 8 diets.

[g]Values represent mean of 6 hearts from each of 7 diets (corn oil not included).

TG fluctuated during the post-weaning period, and finally increased, despite the fact that the solid food available was low in fat (ca. 3 to 5%). Adaptation to the 20% fat diets resulted in even higher levels of cardiac TG regardless of the type of fat or oil, thus confirming previous findings (16).

There was a significant decrease in the cardiac lipid content when weaned rats started to eat the semisynthetic, 20% fat diet. This decrease was evident in the level of cholesterol and all cardiac phospholipids, particularly CPG. On continued feeding, the major cardiac phospholipids (CPG, EPG and DPG) and cholesterol increased to the fourth week; the values at 16 weeks, reported previously (8), were lower than at four weeks. This change in content of cardiac phospholipids and cholesterol with age was consistent with that reported by Nagai et al. (15), who also observed a decrease in cardiac cholesterol, CPG and EPG of rats from 20 to 40 days of age and then a subsequent increase. However, we did not observe the concomitant 4-fold increase in serine phosphoglyceride (SPG) plus SP reported by these workers. Furthermore, the experiment did not last long enough to test reported increases in cardiac cholesterol, SP and DPG at 33 mo of age (17). If the cardiac lipid classes were expressed as percent of total lipids, instead of mg/g wet weight (Table 3), the cardiac lipid profile was more constant. A constant lipid profile would be expected of membrane lipids because the function of the tissue remains the same (18).

Fatty Acid Changes in CPG and EPG

Analyses of the fatty acid composition of cardiac phospholipids are summarized in Figures 1 and 2. The results for corn oil are presented in the same plots as the results for LEAR based oils.

The relative concentration of total saturated fatty acids was higher in cardiac CPG than in EPG (Fig. 1), with 18:0 being the major saturated fatty acid at 30 to 34% in each of the two phospholipids. The concentration of 16:0 was higher in CPG (15-18%) than in EPG (6-7%). An increase in dietary saturates by addition of cocoa butter (56% saturates) significantly increased the relative concentration of total saturates in both phospholipids. On the other hand, the addition of triolein, which diluted the content of dietary saturates, reduced the relative concentration of total saturates in both phospholipids, but not significantly. These changes generally were observed after one week on the diets, maintained throughout the next four weeks and also were evident in the 16-week results. The increase in total saturates in cardiac CPG and EPG occurred mainly in 18:0, despite

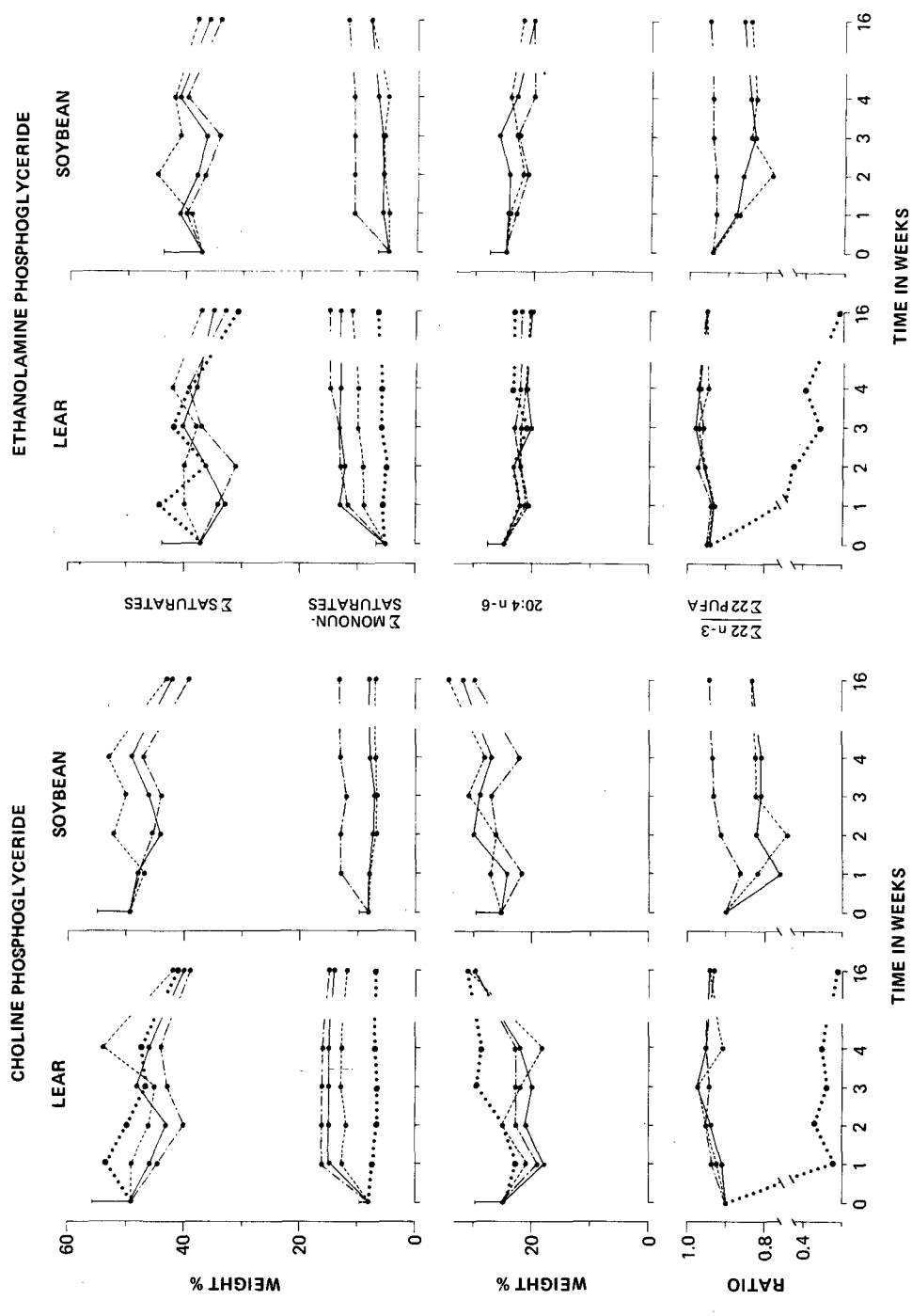

FIG. 1. Relative distribution of fatty acids in heart choline and ethanolamine phosphoglycerides of male rats fed the experimental diets from weaning (time 0) to 16 weeks. The rats were fed diets containing LEAR or soybean oil (——); these oils supplemented with cocoa butter (- - -), triolein (—·—·—) or corn oil (· · · ·). The vertical bar at time 0 represents the least significant difference ($P<0.01$) obtained from error estimates of analysis of variance from the 1- to 4-week results.

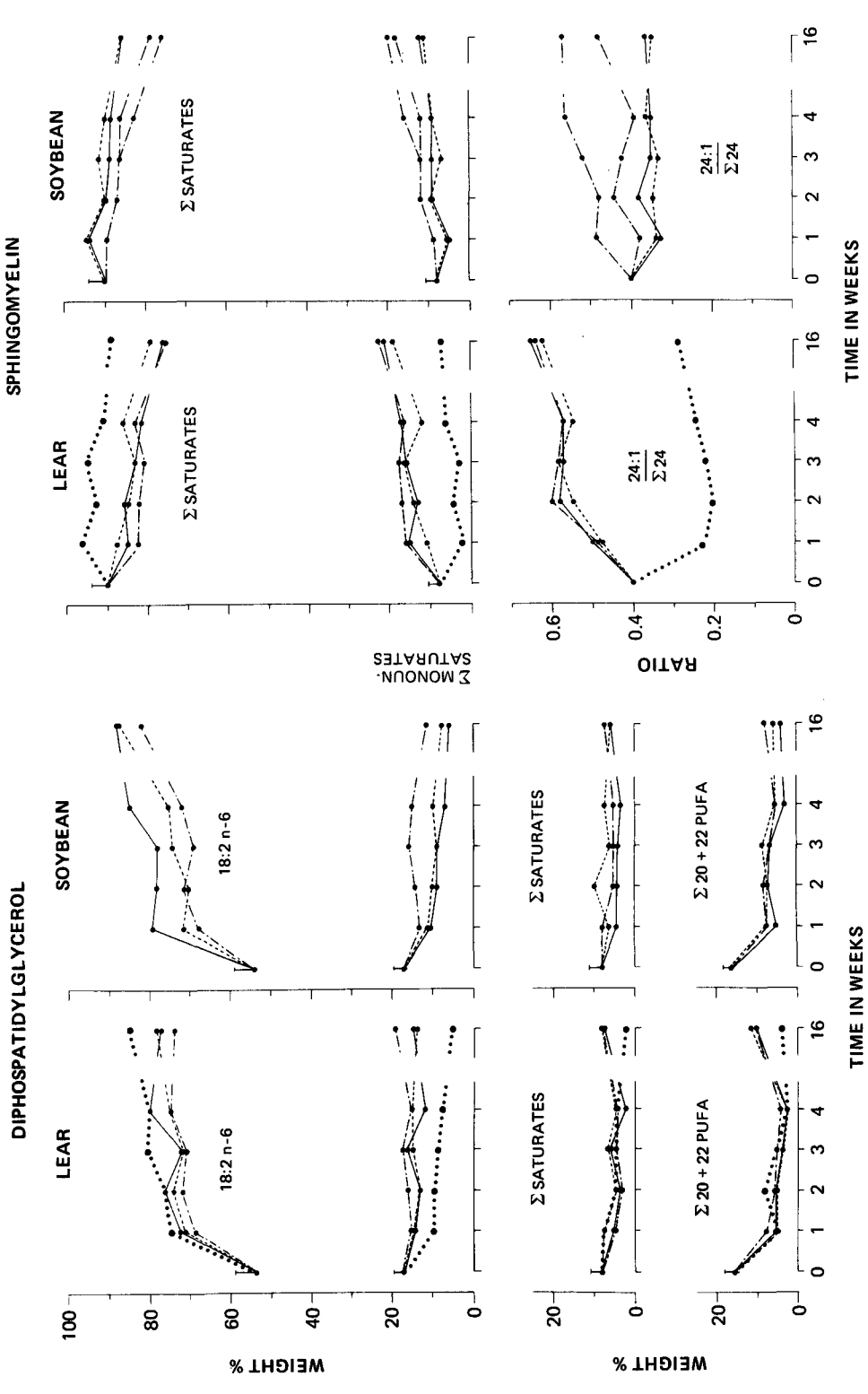

FIG. 2. Relative distribution of fatty acids in heart diphosphatidylglycerol and sphingomyelin (SP) of male rats fed the experimental diets from weaning (time 0) to 16 weeks. For details see Fig. 1. There are two lines with the same symbol (–·–·–) in the SP soybean graph representing the two soybean oil-triolein mixtures, with and without long chain monounsaturated fatty acids.

the fact that the addition of cocoa butter (26% 16:0, 29% 18:0; Ref. 8) and the dilution of saturates with triolein affected the content of dietary 16:0 and 18:0 about equally. The levels of total saturates were somewhat lower in the 16-week data than in the 1- 4-week data for both CPG and EPG.

The sum of the cardiac monounsaturates was relatively low in both phospholipid classes, EPG being generally lower than CPG (Fig. 1). Diets rich in monounsaturates, such as the LEAR oil-based diets and the soybean oil-triolein mixtures, gave a marked increase in the content of monounsaturates of CPG and EPG within the first week on diet, and these levels were maintained throughout the feeding trial (Fig. 1). The rapid turnover of fatty acyl chains in cardiac CPG and EPG within the first week is consistent with the results of Innis and Clandinin (19), who observed a half-life of two to three days for the major fatty acids.

The relative concentration of arachidonic acid (20:4 n-6) in cardiac EPG showed no significant diet or time effect (Fig. 1). In cardiac CPG, the relative concentration of 20:4 n-6 was significantly lower by the fourth week in rats fed LEAR oil-based diets compared to rats fed soybean oil- or corn oil-based diets. However, no significant differences were observed among all diets at 16 weeks (8). The increase of 20:4 n-6 in CPG at 16 weeks was at the expense of 18:2 n-6. These results are consistent with those of Gudbjarnason (29), who observed a 9% increase of 20:4 n-6 in cardiac CPG at the expense of 18:2 n-6 in rats two vs 18 mo of age, while the level of 20:4 n-6 in EPG was not altered with age. The cardiac tissue appears to maintain a consistency of composition which reflects its uniformity of function. The relative constancy of 20:4 n-6 is evident in many studies which report the composition of phospholipids in such tissues as the heart (19-23) and brain (24,25), but the liver, being a rapidly metabolizing organ, shows much less constancy of 20:4 n-6 levels (22,26-28).

The content of the C22 PUFA was much higher in cardiac EPG (20-28%) than in CPG (4-8%), and higher at weaning and at 16 weeks than during the 1-4 week period. Among the C22 PUFA, the n-3 PUFA (22:5 n-3 and 22:6 n-3) predominated in the cardiac phospholipids of the weaned rat (Fig. 1). The ratio of the C22 n-3 PUFA to the total C22 PUFA depended on the dietary content of 18:2 n-6 and 18:3 n-3. It was high when the dietary oil contained about 7-10% 18:3 n-3 and 20% 18:2 n-6 as in LEAR oil or in soybean oil-triolein mixtures. When the dietary content of 18:2 n-6 was raised to >40% in the presence of the same amount of 18:3 n-3, the ratio decreased only to 0.8. However, when the content of 18:3 n-3 was <1%, the ratio reduced drastically to <0.4% as with corn oil, and 22:5 n-6 predominated (Fig. 1). Increasing dietary saturates had no noticeable effect. Again, changes in the C22 PUFA of cardiac EPG and CPG occurred within the first week on diet and were maintained thereafter.

Fatty Acid Changes in DPG

The predominant fatty acid in DPG was 18:2 n-6 (Fig. 2). The relative concentration of 18:2 n-6 in all dietary groups showed a significant linear trend to the fourth week at the expense of the C20 and C22 PUFA. Large differences in dietary 18:2 n-6 (LEAR and corn, Table 1) appeared to have little effect on the 18:2 n-6 content of DPG. An increase in dietary saturates and monounsaturates led to increases in [16:0 and 18:0], and 18:1, respectively, for cardiac DPG. Some of the dietary oils in this study contained small amounts of 22:1 n-9, i.e., 0.4% and 0.6% (see ref. 8 for complete fatty acid composition of diets). There was no apparent accumulation of 22:1 n-9 in cardiac DPG like that observed when dietary oils rich in 22:1 n-9 (>25%) were fed to rats (30-32).

Fatty Acid Changes in SP

Cardiac SP consisted primarily of long chain saturated fatty acids (Fig. 2) ranging from 16:0 to 24:0, including minor amounts of odd chain fatty acids from 17:0 to 23:0. Stearic acid (18:0) was the major fatty acid (20-30%) followed by 22:0 (15-25%), 16:0 (10-20%), 20:0 (10-20%) and 24:0 (5-15%). While the overall relative concentration of total saturates decreased with time among the diets, this was due mainly to 16:0 and 18:0, because 24:0 increased and 20:0 and 22:0 remained constant. Corn oil-fed rats had consistently the highest level of total saturates in cardiac SP, but this result could not be attributed to higher levels of dietary saturates in this dietary oil (Table 1).

The total monounsaturates in cardiac SP showed a significant linear increase up to four weeks in all the LEAR oil and soybean oil groups (Fig. 2). The presence of long chain monounsaturates greater than 18:1 in the dietary oils resulted in a significant increase in total monounsaturates in cardiac SP, specifically nervonic acid (24:1 n-9). This was particularly evident when comparing the two diets containing soybean oil-triolein mixtures, with and without added long chain monoenes (both diets are included in Fig. 2 with the same broken line symbol). The diet with long chain monoenes

gave significantly higher levels of total monounsaturates, specifically 24:1 n-9, and gave a 24:1 to Σ24 (24:0 plus 24:1) ratio similar to that observed for all the LEAR oil-based diets. The results indicated little accumulation of 20:1 and 22:1 in cardiac SP, but a marked increase in 24:1, which suggests that 20:1 and 22:1 were chain elongated to 24:1. This is in agreement with studies using mouse brain (33) and rat lung (34), in which 24:1 n-9 rather than 22:1 n-9 accumulated following administration of 22:1 n-9.

The fatty acid composition of cardiac SP from corn oil-fed rats was significantly different from both the soybean oil and LEAR oil-based groups. The content of total saturates of cardiac SP was highest in the corn oil-fed group, and this was due mainly to higher levels of 16:0, 20:0 and 24:0.

Alkenyl Ether Changes in CPG and EPG

In the weaned rat, 16:0 was the major chain length in both phosphoglycerides, with 18:0 and 18:1 higher in EPG than in CPG (Table 4). Except for the corn-oil fed rats which showed only slightly higher levels of 18:2, all other dietary groups had markedly altered alkenyl ether compositions. The changes appeared to plateau after three to four weeks on diet, and for this reason an average of these two time periods is presented in Table 4. These trends contrast with fatty acid changes shown in Figures 1 and 2, which were essentially completed within the first week on test.

Diets rich in 18:1 fatty acids, such as the LEAR oil-based diets and soybean oil-triolein mixtures, resulted in greatly increased 18:1 alkenyl ether at the expense of 16:0. The magnitude of the changes was greater in EPG than in CPG. Increasing the saturated fatty acids in the diet resulted in increased levels of 18:0 alkenyl ethers at the expense of 18:1. The increase in only the C18 chain length, despite the fact that both 16:0 and 18:0 fatty acids were raised about equally by the addition of cocoa butter in the diet, was consistent with the fatty acid changes in the cardiac phospholipids. No long chain monoalkenyl ethers greater than 18:1

TABLE 4

Composition of the Alkenyl Groups of Choline and Ethanolamine Phosphoglycerides of Hearts of Rats Fed Experimental Diets for 3 and 4 Weeks (average) and After Weaning[a]

	Chain length: number of double bonds						
Dietary oils[b]	15:0	16:0	17:0	18:0	18:1 n-9	18:1 n-7	18:2
Choline phosphoglyceride							
Weaned rat	1.0	81.5	0.9	6.3	7.6	1.3	0.9
Corn	1.2	80.0	0.7	6.1	8.7	1.1	2.0
LEAR	0.9	64.1	0.9	7.0	23.2	2.5	1.2
LEAR + CB	0.8	61.2	0.7	12.9	21.6	1.5	0.7
LEAR + 18:1	0.6	60.5	0.6	7.2	27.5	2.2	1.2
Soybean	0.8	75.8	0.9	9.0	10.3	1.4	1.8
Soybean + CB	0.5	73.3	0.9	13.0	9.9	1.2	1.2
Soybean + 18:1	1.0	65.4	1.1	8.1	21.1	1.7	1.3
Soybean + 18:1 + LCM	0.9	61.4	1.5	7.8	25.4	1.6	1.3
Ethanolamine phosphoglyceride							
Weaned rat	0.2	56.1	1.2	22.8	14.4	3.5	1.4
Corn	0.4	52.3	0.9	24.9	17.5	1.3	2.7
LEAR	0.1	27.9	0.6	22.5	42.2	5.7	1.0
LEAR + CB	0.1	31.4	0.6	34.4	28.0	4.1	1.4
LEAR + 18:1	0.1	27.8	0.8	23.4	41.9	5.0	1.0
Soybean	0.1	41.2	1.1	31.6	19.0	3.8	3.2
Soybean + CB	0.1	38.7	0.7	41.2	15.3	2.8	1.2
Soybean + 18:1	0.1	30.5	1.2	28.0	36.8	2.3	1.1
Soybean + 18:1 + LCM	0.2	27.9	1.4	26.4	38.7	3.5	1.9

[a]The alkenyl groups were analyzed as their cyclic acetals of 1,3-propanediol (12) by GLC. The alkenyl ethers of all rats on each diet and each time period were pooled. Only the results of weaned rats and an average of the 3- and 4-week data are presented; the latter were similar. Weaned rats represent the mean of 6 pairs of rats. The other values are an average of 3 pairs of rats/diet/time period.

[b]LEAR, low erucic acid rapeseed; CB, cocoa butter; 18:1 triolein; LCM, long chain monenes (20:1, 22:1, 24:1).

were detected in the cardiac lipids of any dietary group.

Factors Which Appear to Relate to Focal Heart Lesions

In previous publications it was demonstrated that the incidence of focal heart lesions in male rats fed high fat diets was directly related to continuous feeding of the dietary fats (1,35), and negatively correlated to the concentration of saturated fatty acids in the dietary fat (4). Experimental evidence for this correlation was provided in a study specifically designed to test the effect of increasing dietary saturates (7,8), and the conclusions subsequently have been confirmed (36). The results of this study identified two major lipid patterns which appear to be highly correlated to focal heart lesions.

The relative concentration of the C22 n-3 PUFA (especially 22:6 n-3) was highest in rats fed the LEAR oil-based diets, slightly lower in rats fed the soybean oil-based diets, and much lower in corn oil-fed rats (Fig. 1). This order is basically the same as the incidence of focal heart lesions in male rats fed these vegetable oils (1). However, no differences in the proportion of cardiac C22 n-3 PUFA were observed in the 1-4-week (Fig. 1) and 16-week (8) results, even though manipulations in dietary saturates were effective in reducing the incidence of focal heart lesions (7,8). Therefore, it seems that the C22 n-3 PUFA are not the only indicators of cardiopathogenicity.

The sums of saturates in cardiac phospholipids were positively correlated with the levels of dietary saturates and negatively correlated with the incidence of focal heart lesions (Table 5). The correlations were of similar magnitude during the 1-4-week period and at 16 weeks, the only time at which the cardiopathology was determined. Cardiac DPG appeared to be most resistant to change, showing a weaker correlation with dietary saturates than all the other phospholipids. This is in agreement with studies by Hostetler et al. (37) showing that the fatty acids of DPG, a lipid component found predominantly in the inner mitochondrial membrane (38), are slow to exchange with exogenous sources (39,40). It may be speculated that dietary saturates, which influence the saturated content of cardiac phospholipids, might in turn affect membrane structure and function. On the other hand, the mitochondria, which are rich in DPG, a component more resistant to dietary changes, might be expected to maintain its structure and function. Indeed, cardiac mitochondria of rats were found to be functioning normally (41-44), despite the fact that these dietary fats and oils gave a wide difference in the incidence of focal heart lesions when fed to male rats at high levels (1).

The relative concentration of total monounsaturates in the cardiac phospholipids was positively related to dietary monounsaturates, particularly in CPG, EPG and the 24:1/Σ24 ratio in SP. Based on these results, one would expect a positive relationship of total cardiac monoenes to incidence of focal heart lesions. In fact, the pure oils also follow the same order of increasing monounsaturated fatty acid content and increasing incidence of focal heart lesion (1). However, the results indicate that the significant changes in cardiac monounsaturates associated with the addition of triolein which were evident in the first week and maintained throughout the 16 weeks (Fig. 1 and 2), did not

TABLE 5

Correlation Between Sum of Saturates in Cardiac Phospholipids at 1 to 4 Weeks and 16 Weeks and Dietary Saturates, and Between Sum of Saturates in Cardiac Phospholipids at 16 Weeks and Incidence of Focal Heart Lesions

Phospholipids[a]	Time rats were fed experimental diets (weeks)	Correlation to dietary saturates	Correlation to heart lesion incidence[b]
CPG	1-4	+ 0.84	
	16	+ 0.93	− 0.77
EPG	1-4	+ 0.91	
	16	+ 0.97	− 0.80
SPG + IPG	1-4	+ 0.85	
	16	+ 0.89	− 0.74
SP	1-4	+ 0.93	
	16	+ 0.84	− 0.45
DPG	1-4	+ 0.51	
	16	− 0.72	+ 0.25

[a]For abbreviations of phospholipids see footnote a, Table 3.

[b]See reference 8 or incidence of focal heart lesions.

produce a concomitant increase in the incidence of focal heart lesions (8).

In conclusion, the results of this study suggest two major fatty acid changes which appear to contribute to the development of focal heart lesions in male rats, particularly the fastest growing rats in the group (45). Saturates and the C22 PUFA of the linolenic acid family showed a relationship to the incidence of this lesion, and appear to act in concert. The fatty acid changes occurred very early (within the first week) and were maintained throughout the experimental period. A continuous exposure to an altered cardiac fatty acid composition appears to develop these focal heart lesions. This supports the concept that these lesions are the result of a nutritional rather than a toxicological problem (1,46).

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Esterified Lipids of the Freshwater Dinoflagellate *Peridinium lomnickii*

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ABSTRACT

Steryl esters, phytyl esters and triacylglycerols of a naturally occurring freshwater dinoflagellate, *Peridinium lomnickii*, were identified using capillary gas chromatography-mass spectrometry (GC-MS). Steryl esters differing in degree of unsaturation were separated, prior to analysis, by argentation thin layer chromatography. 5α(H)-Cholestanol was more dominant, relative to 4α-methylstanols, in steryl esters than in the free sterols, but the same sterol moieties occurred in both fractions. Monoenoic fatty acids were enriched in the steryl esters relative to the free fatty acids. Major acyl groups in steryl esters were 16:0 or 20:1, with smaller amounts of 14:0 and 18:1. In triacylglycerols the acyl moieties were 14:0, 16:0, 18:1, 16:1 and 12:0, in order of decreasing abundance. Phytyl esters, previously inferred to occur in a marine dinoflagellate only by analysis of transesterified products, were identified by GC-MS comparison with authentic compounds. Direct analysis of these esterified lipids has not been reported for freshwater phytoplankton. The 4α-methylstanyl esters, 5α(H)-cholestan-3β-yl esters and phytyl esters occurring in *P. lomnickii* are further features in common with marine dinoflagellates, additional to the 4α-methylsterols reported previously.
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INTRODUCTION

Interest in the sterols of dinoflagellates has focussed on the wide variety of structures (including unusual side-chain alkylation), the presence of 4α-methyl nuclear-saturated sterols, an aspect in which dinoflagellates differ from other classes of marine and freshwater algae, and the implications of these features for sterol biosynthesis (1). The restricted occurrence of 4α-methylsterols in nature has led to their use as biological markers of dinoflagellate input to recent marine (2-6) and lacustrine (7) sediments. Both free and esterified 4α-methylsterols present in Black Sea sediment were thought to reflect the sterol composition of contributing source organisms (3). Evidence is accumulating that steryl esters may occur widely in dinoflagellates (8-10), but the saponification step often used to facilitate analysis (9,10) prevents recognition of the individual constituents; the presence of phytyl esters in a marine dinoflagellate also has been inferred by analysis of transesterified products (11). The molecular composition of triacylglycerols, the primary metabolic energy reserve lipid of most phytoplankton (12), also cannot be determined after saponification.

The development of on-column injection for capillary gas chromatography (GC), the use of improved interfaces between the GC and mass spectrometer and bonded-phase GC columns of high thermal stability now enable structural information to be obtained directly on lipids of low volatility. Capillary GC-MS therefore has been used to study esterified lipids of a freshwater dinoflagellate, *Peridinium lomnickii* Woloszynska. We have identified steryl esters containing the 4α-methylsteroid moieties characteristic of the free lipids of dinoflagellates and also directly analyzed phytyl esters obtained from the organism. Geochemical implications of these results are discussed briefly.

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EXPERIMENTAL

Materials

Redistilled analytical grade solvents were used; Analar grade KOH and aq HCl were used without further purification. No contamination from these sources was evident in control experiments.

Authentic steryl esters (Table 1) obtained from Nu Chek Prep, Elysian, Minnesota, and hexadecyl stearate (Analabs, New Haven, Connecticut) were >98% pure by GLC analysis. Phytyl hexadecanoate and octadecanoate were synthesized from phytol (Analabs) by the procedure of Gellerman et al. (13) and purified by TLC on silica gel G.

Isolation and Extraction

Peridinium lomnickii was collected from the stratified water column of Priest Pot, an eutrophic lake in the English Lake District (area ca 10^4 m^2, max depth 3.6 m), by pumping

water from a layer 1.2 m below the surface, corresponding to a maximum chlorophyll *a* concentration (>1 mg l^{-1}) associated with this alga; further concentration was achieved by centrifuging. The dinoflagellate comprised >95% of the sample. Lipids were extracted by successive treatment of wet algae (ca 8 g, equivalent to 0.95 g dry weight) with 30 ml portions of chloroform/methanol (1:2, v/v), chloroform/methanol (1:1, v/v) and finally with chloroform at room temperature. The combined extracts were diluted with water (30 ml) to give a chloroform/methanol/water ratio of 2/2/1.8, v/v/v; the chloroform layer was then separated, dried and evaporated. Residual lipids were removed by extraction with chloroform under reflux (Soxhlet) and combined with the former extract, giving total lipids (68 mg). Free fatty acids (2 mg) were removed by chromatography on a column (8 cm × 1.2 cm i.d.) of KOH-impregnated silica gel (3 g). Elution with diethyl ether gave neutral lipids together with chlorophyll pigments; the latter were removed from the eluate by washing with ice-cold aq HCl (1:1, v/v). The neutral lipids (60 mg) were separated on a 1.0 cm i.d. glass column packed to a depth of 13 cm with silica gel (60-120 mesh). After elution with hexane (20 ml) and hexane/diethyl ether (9:1; 15 ml), further elution with hexane/ether (9:1, 20 ml) gave a product which was separated by thin layer chromatography (TLC) on pre-eluted silica gel G, developing with hexane/diethyl ether/acetic acid (89:10:1, v/v/v). Bands corresponding in mobility with authentic samples of hexadecyl stearate (R_f 0.7-0.8) and glyceryl tristearate (R_f 0.13-0.26) were removed and separately eluted.

Wax esters were further resolved by TLC on silica gel G containing 10% w/w of silver nitrate, developing with hexane/ether (9:1) to give four bands (A-D in order of decreasing R_f) corresponding in R_f values with the following authentic compounds, denoted by alkyl/steryl–acyl pairing: 16:0–18:0 ester (band A, R_f 0.75-0.80), C_{29}-Δ^5-stenol–18:0 ester (band B, R_f 0.70-0.75), C_{27}-5α(H)-stanol–16:1ω7, C_{27}-Δ^5-stenol–16:1ω7 and 16:0–18:1ω9 esters (band C, R_f 0.33-0.45), and C_{27}-5α(H)-stanol–18:2ω6, together with more highly unsaturated constituents (band D, R_f <0.30).

Gas Chromatography and Mass Spectrometry

A fused silica capillary column (14 m × 0.32 mm i.d.), coated with DB-1 (0.1 μm) and temperature programmed from 180 C to 320 C at 4 C/min using H_2 at 0.3 kPa inlet pressure as carrier gas, was used for alkyl and steryl esters; corresponding conditions for triglycerides were: 200-325/4 C/min using H_2 at 0.45 kPa. A "dropping needle" injection system (14) and flame ionization detection were used. Quantitative analyses were obtained by peak area integration and comparison with authentic compounds.

Constituents were identified by computerized gas chromatography-mass spectrometry (C-GC-MS) using the above column in a Carlo Erba 5160 chromatograph fitted with an on-column injector, using helium carrier gas and coupled to a Finnigan 4000 quadrupole filter mass spectrometer operating in the EI mode. The ion source was operated at 40 eV with an ionization current of 350 μa. Mass spectral data were acquired and edited using an INCOS 2300 data system, scanning from m/z 50 to m/z 700 in 1.5 sec. EI spectra of triacylglycerols also were obtained by direct insertion probe analysis.

RESULTS

Alkyl and Steryl Esters

GC analyses of authentic steryl and alkyl esters showed that steryl esters, having cholesterol or the related 24-methyl and 24-ethyl-Δ^5-sterols linked to saturated straight chain acids, eluted just before the *n*-alkyl ester containing three more carbon atoms (15). For steryl esters having the same acyl group, the elution order was analogous to that of sterol trimethylsilyl-ethers (3). Resolution of the Δ^5-stenyl and corresponding 5α(H)-stanyl esters containing the same acyl group was about 0.7 under the GC conditions used, but the compounds were unresolved when the same column was operated under GC-MS conditions, hence separation of Δ^5-stenyl from 5α(H)-stanyl esters by argentation TLC gives GC peaks having simplified mass spectra.

EI mass spectra of steryl esters (Table 1), as recently reported (16), do not show acyl fragment ions analogous to those in alkyl esters; also, the base peak is a function of both the acid and sterol moiety. 5α(H)-stanyl esters of saturated acids gave EI mass spectra showing a strong fragment ion consistent with loss of RCOOH and charge retention by the sterol moiety, together with structurally diagnostic fragmentation analogous to that of steryl acetates (17). The absence of unsaturation in the sterol nucleus of desmethylstanols is thus reflected by fragment ions at m/z 215, 257 while the presence of a 4α-methyl substituent gives fragment ions at m/z 229 and 271, as in sterol derivatives usually used for GC-MS (3). Analogously, esters of dinosterol showed a major fragment ion at m/z 69, characteristic of sterols having a Δ^{22}-23,24-dimethyl side chain and an ion at m/z 139 indicative of a C_{10} monoene (or cyclopropanoid)

TABLE 1

Electron Impact Mass Spectral Data for Steryl and Phytyl Esters

Ester[a]	M^+	Fragment ions[b]
5α(H)-cholestan-3β-yl–16:1ω7	624(0.7)	371(100), 370(62), 355(25), 316(22), 257(28), 215(49), 203(29), 149(40)
Cholest-5-en-3β-yl–16:1ω7	622(0.02)	368(100), 353(21), 260(22), 255(22), 247(28), 213(15), 147(37)
5α(H)-cholestan-3β-yl–18:2ω6	650(0.3)	371(57), 370(60), 355(30), 316(16), 257(27), 215(89), 149(77), 57(100)
Phytyl hexadecanoate	534(1)	351(0.8), 324(1.5), 296(5), 278(35), 239(8), 123(83), 97(78), 82(86), 68(84), 57(100)
Phytyl octadecanoate	562(1)	379(0.5), 352(1.5), 296(5), 278(38), 267(5), 123(78), 95(77), 82(81), 68(78), 57(100)

[a]Spectra of steryl esters obtained using direct insertion probe; those of phytyl esters by GC-MS.

[b]Figures in parentheses give ion abundance as % of base peak in range m/z 50-650.

side chain. In Δ^5-stenyl esters having saturated (16) or monounsaturated (Table 1) acyl groups, M-RCO_2H was the base peak. Direct insertion EI probe analysis of authentic 5α(H)-cholestanyl esters of unsaturated acids (Table 1) gave mass spectra characterized by a strong fragment ion at m/z 371, corresponding with M-RCO_2, in addition to that due to the $(M\text{-}RCO_2H)^+$ stanol moiety; Lusby et al. (16) recently reported similar results. These observations aided recognition of unsaturation in the acyl group of 5α(H)-stanyl esters obtained from *P. lomnickii*, in the absence of acyl fragment ions. However, acyl unsaturation also was evident from the marked effect on the R_f value during argentation TLC. The presence, in addition to M-RCO_2H ions, of weak M^+ ions in spectra of 5α(H)-stanyl and Δ^5-stenyl esters obtained by direct probe analysis and by GC-MS, enabled the size of the acyl group in constituents of *P. lomnickii* to be deduced; Lusby et al. (16) did not observe M^+ ions in EI mass spectra of these steryl esters.

EI mass spectra of phytyl esters also show a strong M-RCO_2H fragment ion (Table 1). A weak M-210 ion corresponds with the loss of a C_{15} monoene by cleavage of the allylically-activated carbon-carbon bond in the phytyl chain. Weaker fragment ions, structurally diagnostic for branching in the phytyl chain, occur at M-183, M-113 and M-43.

Band A (600 μg), obtained by argentation TLC of the wax/steryl esters of *P. lomnickii*, consisted of saturated alkyl and 5α(H)-stanyl esters (1:2.2, w/w); the molecular composition of the latter is shown in Table 2.

Band B (160 μg) contained alkyl and steryl esters in the proportions 4.5:1 (w/w); the steryl esters are listed in Table 2. The alkyl esters eluted just in front of even carbon number saturated straight chain C_{30}-C_{42} esters and were each characterized by a fragment ion at m/z 278 and a molecular ion 26 mass units higher than that of the next eluting straight chain ester. The fragmentation in the range m/z 50-150 closely resembled that of neophytadiene (18), suggesting that these constituents are esters of phytol (3,7,11,15-hexadec-2-en-1-ol) with C_{12}-C_{24} fatty acids respectively; only the C_{14}, C_{16} and C_{18} esters occurred in more than trace amounts (20, 55, and 10 μg, respectively). The identities of phytyl hexadecanoate and octadecanoate were confirmed by GC co-elution and mass spectral comparison with synthetic samples prepared according to Gellerman et al. (13). Two additional constituents eluting between the C_{16} and C_{18} esters of phytol and present in significant amounts also showed mass spectra containing a strong fragment ion at m/z 278. The first eluting major constituent (10 μg) tentatively was identified as phytyl phytanate; the minor constituent was not identified.

Band C (500 μg) contained C_{30}-C_{36} monoenoic alkyl esters (110 μg) and steryl esters of 18:1 and 20:1 acids (390 μg), major constituents of which are listed in Table 2.

The sterol composition of the esters is compared with previously published data (7) for the free sterols in Table 2. The fatty acid distribution in esters of the major sterols and that of the free fatty acids are shown in Table 3.

Band D (200 μg) was not analyzed by GC-MS; GC elution data and TLC mobility suggested the presence of polyenoic alkyl and steryl esters.

Triacylglycerols

Triacylglycerols in the range C_{40}-C_{52} were detected by GC analysis. In the absence of stan-

TABLE 2

Steryl Esters of *P. lomnickii* Identified by GC-MS

Sterol	Acyl chain[a]	Amount (μg)	Sterol abundance[b]	
			Esterified[c]	Free[d]
Band A[e]				
5α(H)-cholestan-3β-ol	14:0	40	45	21
	16:0	100		
4α-Methyl-5α(H)-cholestan-3β-ol	14:0	25	23	17
	16:0	57		
4α,24-Dimethyl-5α(H)-cholestan-3β-ol	14:0	18	7	9+
	16:0	38		
4α,23,24-Trimethyl-5α(H)-cholest-22E-en-3β-ol	14:0	14	10	18x
	16:0	50		
4α,23,24-Trimethyl-5α(H)-cholestan-3β-ol	14:0	20	11	13
	16:0	43		
Band B[e]				
Cholest-5-en-3β-ol	14:0	2	1	tr
	16:0	6		
24-Ethylcholest-5-en-3β-ol	16:0	7	1	5
4α,24-Dimethyl-5α(H)-cholest-22E-en-3β-ol	16:0	3	tr	3
4α,23,24-Trimethylcholesta-5,22-dien-3β-ol	16:0	7	1	13x
Band C[e]				
5α(H)-cholestan-3β-ol	18:1	85		
	20:1	124		
4α-Methyl-5α(H)-cholestan-3β-ol	18:1	24		
	20:1	69		
4α,23,24-Trimethyl-5α(H)-cholest-22E-en-3β-ol	18:1	19		
4α,23,24-Trimethyl-5α(H)-cholestan-3β-ol	18:1	26		

[a]Site or stereochemistry of C=C in acyl group not known.

[b]As percentage composition.

[c]Calculated from combined abundance of esters containing given sterol moiety.

[d]Data recalculated from Ref. 7; +, including 24-ethyl-5α(H)-cholestan-3β-ol; x, overlapping peaks, as argentation TLC not used.

[e]From TLC separation on silica gel containing 10% w/w silver nitrate (see Experimental).

TABLE 3

Relative Abundance of Free and Esterified Fatty Acids in *P. lomnickii*

Sterol	Acyl chain[a]					
	14	16	18:1	18:0	20:1	Others[b]
None (i.e., free acids)	8	47	14	15	3	13
5α(H)-Cholestan-3β-ol	11	29	24	–	36	–
4α-Methyl-5α(H)-cholestan-3β-ol	14	33	14	–	39	–
4α,23,24-Trimethyl-5α(H)-cholest-22-en-3β-ol	16	60	23	–	†[c]	–
4α,23,24-Trimethyl-5α(H)-cholestan-3β-ol	22	48	29	–	†[c]	–

[a]% Abundance.

[b]Including Δ16, 5%, polyunsaturated C_{18}, 4%, $>C_{20}$ saturated, 4%.

[c]Weak peaks in GC, not identified by MS.

dard compounds spanning a wide molecular weight range, the relative abundance of each homologue (Table 4) was estimated assuming an equal response, although weak discrimination of higher triglycerides has been reported (19). Triacylglycerols having the same carbon number and degree of unsaturation but different acyl chain combinations are partially resolved during GC analysis on apolar capillary columns (20), as are triacylglycerols differing in unsaturation; thus, GC-MS was needed to determine the molecular composition.

TABLE 4

Molecular Composition of Triacylglycerols from *Peridinium lomnickii*

Carbon number double bonds	Percentage composition	Acyl combinations[a]
40:0	3	12/14/14
		12/12/16
42:0	17	14/14/14
44:0	17	14/14/16
46:1	10	14/16/16:1
		14/14/18:1
46:0	15	14/16/16
48:1	18	14/16/18:1
48:0	6	16/16/16
50	12	NA
52	2	NA

[a]Saturated; except where shown, major constituent given first. NA not analyzed.

Direct insert EI probe mass spectrometry showed that the triacylglycerols contained 14:0, 16:0, 18:1, 16:1 and 12:0 acyl substituents in order of decreasing abundance, based on the relative heights of the respective $(RCO)^+$ ions diagnostic of these acyl groups (21). The major acyl combinations of each peak were determined using GC-MS (Table 4). However, in the absence of suitable standard compounds, quantitative analysis of molecular composition, based on corrected relative acyl ion intensities, was not possible.

DISCUSSION

Steryl and Phytyl Esters

Steryl esters occur widely in living matter, including higher plants (22), mosses (23), fungi (24,25) and in algae belonging to the Chlorophyta (26), Xanthophyceae (27) and Dinophyceae (1). Steryl esters are believed to function as a reserve for free sterols which are required for membrane building. The sterol composition of dinoflagellate steryl esters often shows a greater complexity than the free sterols (9) or different proportions of the same constituents (10). As steryl esters occur in particulate matter in the oceanic water column (28) and also in marine (3) and lacustrine (15) sediments, they are useful for geochemical assessment of sources and transformations of organic matter in sedimentary environments.

Until recently, analysis of steryl esters involved saponification and identification of the resulting sterols and fatty acids. However, direct analysis by CI GC-MS (29) gave structural data about the sterol and acyl moieties. EI mass spectra, used in this work, also give structurally diagnostic fragmentation for the sterol moiety, as described above. The steryl esters of *P. lomnickii* are composed of sterols present in the free lipids (7) with 5α(H)-cholestanol rather more dominant, relative to 4α-methylsterols, than in the free sterols (Table 2). In *Glenodinium* sp., however, a wider range of 4α-methylsterols occurred as esters than as free sterols; the dominance of dinosterol in the former was markedly reduced (9).

A notable feature of the fatty acid composition of the steryl esters is the enrichment of unsaturated and, to a lesser extent, 14:0, compared with the free fatty acids (Table 3). There appears to be little available comparable data for aquatic organisms; however, an enrichment in 16:0 and 18:3 fatty acids in steryl esters, relative to total lipids, was reported in species of the Xanthophyceae (27).

The occurrence of phytol and its metabolites as alcohol and acid moieties of wax esters has been reviewed (30). Species of moss contain phytyl phytenate in addition to straight chain esters of phytol (13,23). Phytyl esters present in marine zooplankton were attributed to dietary intake of phytol resulting from detrital feeding either on bottom sediments or on non-living suspended particulate material (31). Phytyl esters of predominantly polyenoic acids constituted 5% of total lipids in a cultured marine dinoflagellate *Peridinium foliaceum* (11); the esters were thought to occur in the membrane-bound eyespot structure (32) and to serve as a potential energy reserve or buoyancy aid (11). In *P. lomnickii*, which does not possess an eyespot, phytyl esters were much less abundant, accounting for only 0.2% total lipids. Previously it was found that phytyl esters were destroyed during attempted GC separation on packed columns, but the corresponding phytanyl esters were stable (13); using capillary columns, phytyl esters may be analyzed directly.

Phytol, the ester-linked side chain of chlorophylls *a* and *b*, has been recognized as the precursor of a variety of acyclic isoprenoids having up to 20 carbon atoms detected in aquatic organisms and sediments. Formation of metabolites has been studied by incubation of ^{14}C-labelled phytol in sediments (33), by cultivation of marine bacteria using phytol as sole carbon source (34) and by feeding zooplankton with a unialgal diet containing phytol as sole alcohol (35). As decay of the aquatic moss *Fontinalis antipyretica* resulted in rapid hydrolysis of phytyl phytenate (36), the phytyl esters occurring in higher plants and dinoflagellates may constitute a potential minor source of phytol in the aquatic environment, through breakdown of organic detritus within the food web. Recognition of input derived from *P. lomnickii*

to the lipids of the underlying sediment of Priest Pot, based on comparison of the distribution of free sterols and steroidal ketones, has been reported (7). The major phytyl esters detected in the dinoflagellate, phytyl–C_{16} and phytyl–C_{18}, also were identified in the ester fraction occurring in the surficial sediment of Priest Pot (Cranwell, unpublished observation). Although higher-plant sources of phytyl esters are known (30), a dinoflagellate source is suggested by the absence of phytyl esters in recent sediment from Coniston Water, in which terrestrial input is dominant but 4α-methylsterols are insignificant (Cranwell, unpublished data). Input of phytol to the anoxic zone of an intertidal sediment was found not to be associated with higher plant input, but with the presence of a non-chlorin phytyl ester (37).

Triacylglycerols

The fatty acid composition of algal triglycerides has been reported most frequently for members of the Bacillariophyceae (38-40). Dominant acyl groups were 16:0, 16:1 and 20:5ω3 (38), although some species contained considerable amounts of 20:4 (39). Variations in the acyl composition of triacylglycerols occurred when organisms were grown under different physiological conditions (40). In two photosynthetic dinoflagellates 16:0 was the dominant acyl group in the triacylglycerols (41, 42), with lesser amounts of 14:0, 18:0, 18:1ω9, 18:2ω6 and 22:6ω3, a heterotrophic dinoflagellate showed a markedly different composition in which 14:0 and 12:0 predominated (42).

In *P. lomnickii* the acyl units occurring in triacylglycerols (Table 4) differ from those of steryl esters in the absence of 20:1 and the presence of 12:0 and 16:1 as minor constituents. We were unable to obtain mass spectra of C_{50} and C_{52} triacylglycerols, owing to temperature limitations of the GC-MS transfer lines. As useful GC-MS analysis of triacylglycerols containing polyenoic acyl groups is not yet feasible (29), the detection of polyunsaturated acyl groups characteristic of dinoflagellates (43), such as 22:6, in triacylglycerols would require saponification.

As major primary producers, dinoflagellates are an important element in the aquatic food chain; the lipid content and composition of these organisms consequently is of considerable biochemical and biogeochemical interest. Steryl esters, phytyl esters and triacylglycerols have a role in membrane building and energy storage. The recognition that dinoflagellates contain steryl esters in which 4α-methylsterols are abundant, as in the free sterols, is relevant to the geochemical occurrence of esterified 4α-methylsterols in sediments. Phytyl esters derived from dinoflagellates may be a source of sedimentary phytol, additional to that arising from breakdown of chlorophyll pigments; diagenesis of phytol in sediments produces a range of isoprenoid derivatives, including pristane and phytane which have been used to assess the oxicity/anoxicity of depositional environments (44). Triacylglycerols are biochemically less stable than wax esters, but were detected in a diatomaceous marine bottom sediment (45). The saturated nature of the acyl groups in the sedimentary constituents, when contrasted with a predominance of unsaturation in the triacylglycerols of diatoms, was interpreted as indicating a zooplankton rather than a phytoplankton source (28). The presence of relatively labile lipids in sediments poses important questions concerning the mechanisms whereby such materials escape recycling within the water column.

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Mass Spectra of Pyrrolidides of Oxo, Hydroxy and Trimethylsilyloxy Octadecanoic Acids

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ABSTRACT

Pyrrolidides of 16 isomeric oxooctadecanoic acids and 17 isomeric hydroxyoctadecanoic acids and their trimethylsily (TMS) ethers have been prepared and their mass spectra measured. Comparison of spectra of all the isomers has shown the variation in amide- and substituent-directed fragmentation with position of the substituent on the 18 carbon chain. Five diagnostic ions are present in spectra of most oxo pyrrolidides, and the most important one is formed by cleavage between the carbons α and β to the oxo group on the amide side. Mass spectra of hydroxy pyrrolidides have four diagnostic ions; the principal one in most spectra is formed by α-cleavage at the oxygenated carbon on the terminal methyl side. There are four characteristic ions in spectra of the trimethylsiloxy pyrrolidides, and the two formed by α-cleavage on one or the other side of the oxygenated carbon are the most important. Spectra of the 2-, 3- and 4-substituted isomers are considerably different from those of the other isomers. Mass spectra of pyrrolidides of three specifically deuterated oxo acids and of eight deuterated hydroxy acids and their trimethylsilyl ethers also were recorded and used to confirm the mode of formation of almost all the ions in the spectra. Two unusual hydrogen migrations, from the carbon α to the oxygenated carbon on the terminal methyl side, are involved in fragmentation of the trimethylsilyl ethers. Spectra have been compared with those of the corresponding oxygenated methyl esters.

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INTRODUCTION

A comparison of mass spectra of methyl esters of oxo and hydroxyoctadecanoic acids was one of the first investigations of spectra of a series of isomeric compounds (1). Spectra of 11 of 16 possible oxo esters and of seven of 17 possible positionally isomeric hydroxy esters were reported. In most cases the spectra contained diagnostic ions which showed the position of the carbonyl or hydroxyl groups fairly clearly, but a large number of competing fragmentations occurred (particularly in spectra of oxo esters), and most ions were accompanied by others (of very variable intensity) resulting from loss of 32 atomic mass units (amu) (1,2). The presence of many ions from each ester made it difficult to analyze mixtures of oxo or hydroxy compounds by mass spectroscopy (2, 3). Later spectra of TMS ethers of methyl esters of some C_{18} and all the C_{16} hydroxy acids were reported (4-7). These spectra were relatively simple, and loss of 32 amu from fragments was not observed.

Pyrrolidides of fatty acids have been proposed as a valuable alternative to methyl esters for mass spectroscopy (8). The first investigation of the spectrum of a pyrrolidide was performed on valeric acid pyrrolidide when it was shown, using deuterated derivatives, that the base peak $(CH_2\text{-}CH_2)_2NC(\overset{+}{O}H)CH_2$, m/z 113, is formed by the McLafferty rearrangement (transfer of hydrogen from C-4 to the carbonyl followed by β-cleavage) (9). The mass spectrum of pyrrolidides was used to show the position of unsaturation in monoenoic acids (10), in polyenoic acids (11) and in acetylenic acids (12). Applications of mass spectroscopy of pyrrolidides to determination of fatty acid structure have been reviewed (13,14). The major cleavages occurring in the spectra of the pyrrolidides of 12-hydroxyoctadecanoic acid and of 11-oxooctadecanoic acid were reported (13). There is a strong tendency for charge retention by the amide due to the relatively low ionization potential (15), and ion m/z 113 is the base peak of these pyrrolidides.

Usually there are no ions which contain the portion of the chain from the substituent to the terminal methyl group. This has been regarded as a limitation on the use of pyrrolidides in mass spectroscopy, particularly for epoxy fatty acids and acids with an ether linkage in the chain (16). Spectra of pyrrolidides of hydrooxo and dihydroxy cutin acids and their TMS ethers have been examined and the advantages of using them instead of methyl esters to avoid formation of extra ions by loss of 32 amu discussed (2). There does not seem to have been any other investigation of mass spectra of pyrrolidides of oxygenated fatty acids.

Mass spectra of the pyrrolidides of the 16 isomeric oxooctadecanoic acids and of the 17 hydroxyoctadecanoic acids and their TMS ethers now have been recorded. Modes of formation

of nearly all the ions observed have been proposed and confirmed by examination of the spectra of pyrrolidides of appropriate specifically deuterated acids, including some of tri-[$^2H_3$]methylsilyl ethers.

EXPERIMENTAL PROCEDURES

Preparation of Pyrrolidides

The following procedure was used because preparation at 100 C (8) gave discolored products which required chromatographic purification. Glacial acetic acid (0.1 ml) was added to pyrrolidine (1 ml), the mixture cooled to 0 C, methyl hydroxy or oxooctadecanoate (0.1-0.01 g) added and the solution kept at 25 C for 24 hr. The solution was poured into water, the product extracted with $CHCl_3$ and the extract washed with 1M HCl and with water. Removal of the solvent gave the nearly colorless pyrrolidide, and thin layer chromatography (TLC) (ether) indicated that most products did not require purification. Typical Rf values of oxo pyrrolidides were 0.15, and those of hydroxy pyrrolidides were 0.05. The pyrrolidide of 3-oxooctadecanoic acid was very impure and was chromatographed on Biosil A; elution with hexane-acetone 17:3 gave the pure derivative in 17% yield. The pyrrolidide of 2-oxooctadecanoic acid also was chromatographed (elution with hexane-acetone 19:1), as were those of 4- and 5-hydroxyoctadecanoic acids (elution with hexane-acetone 7:3); the yields were about 85%. The methyl oxo and hydroxyoctadecanoates were prepared previously (17). The sample of 4-hydroxy ester was mainly γ-lactone and that of the 5-hydroxy ester was partly the free acid, but amides were obtained satisfactorily. Synthesis of the deuterated esters was as follows: methyl 11-oxo[8-$^2H_2$]- and 11-oxo[14-$^2H_2$]octadecanoates (18), 17-hydroxy[17-^{2}H]- and 17-hydroxy[16,18-$^2H_5$]octadecanoates (19) and 8-hydroxy[2-$^2H_2$]-, 8-hydroxy[5-$^2H_2$]-, 11-hydroxy[8-$^2H_2$]- and 11-hydroxy[14-$^2H_2$]octadecanoates (20). Methyl 11-hydroxy[10,12-$^2H_4$]octadecanoate was synthesized from the oxo ester: methyl 11-oxooctadecanoate (0.25 g) was refluxed in CH_3OD (10 ml) containing DCl (35%) in D_2O (0.1 ml) for 18 hr, the solution was diluted with D_2O (50 ml) and the product extracted with $CHCl_3$. The oxo ester obtained was reduced in CH_3OD (10 ml) with $NaBH_4$ (0.04 g) at 20 C for 30 min and the hydroxy ester isolated. Methyl 13-hydroxy [15-$^2H_2$]octadecanoate was synthesized in the same way as 13-hydroxy[16-$^2H_2$] ester (18,20) but starting from [1-$^2H_2$]butanol. TMS ethers were prepared by treating the hydroxy pyrrolidides, in methylene chloride, with bis(trimethylsilyl) acetamide. The TMS ether of the pyrrolidide of 2-hydroxyoctadecanoate was formed very slowly under these conditions and was obtained by heating a pyridine solution of the pyrrolidide with the reagent at 50 C for 4 hr. Tri[$^2H_3$]methylsilyl ethers were prepared by treating the hydroxy derivatives with tri[$^2H_3$]-methylsilyl chloride (MSD Isotopes, Dorval, Quebec) in methylene chloride.

Mass Spectroscopy

Electron impact mass spectra were obtained with a model 4000 Finnigan gas chromatography-mass spectroscopy (GC-MS) system interfaced with a model 2300 Finnigan Incos data acquisition system. The source temperature was 250 C, and the ionization voltage was 70 eV. The GC column was fused silica (20 m × 0.32 mm) coated with DB-1; the linear velocity of the carrier gas (helium) was 40 cm/s. Samples were injected at a temperature of 50 C; the temperature was raised at once to 125 C and programmed at 10 C/min to 250 C.

RESULTS AND DISCUSSION

The descriptions of the spectra have been limited to the four or five important ions, which indicate structure, and a few others including the base peak and the molecular ion. Ions characteristic of the pyrrolidide grouping only, such as ions m/z 70 and 98 and the series of weak ions beginning at ion m/z 126 and increasing by 14 amu, which appear in spectra of most fatty acid pyrrolidides (8,13), are not specifically discussed here. In considering the spectra, the concepts of amide directed fragmentation (ADF), often with hydrogen abstraction, in which fragmentation is dominated by the amide group, and substituent directed fragmentation (SDF), in which it is markedly affected by the substituent (12), have been found very useful.

Pyrrolidides were prepared by the acetic acid-catalyzed reaction of methyl esters with pyrrolidine (8,10) but at 25 C rather than at 100 C. All the derivatives were obtained in good yield except for that of 3-oxooctadecanoic acid, which apparently decomposed extensively and was obtained in only 17% yield.

Mass Spectra of Pyrrolidides of Oxo-octadecanoic Acids

Formation of the diagnostic ions in spectra of oxo pyrrolidides is shown in Figure 1, and intensities of these ions and of the molecular ion and the base peak are listed in Table 1. Variation in intensity of these ions with position of the oxo group is shown by the spectra of the

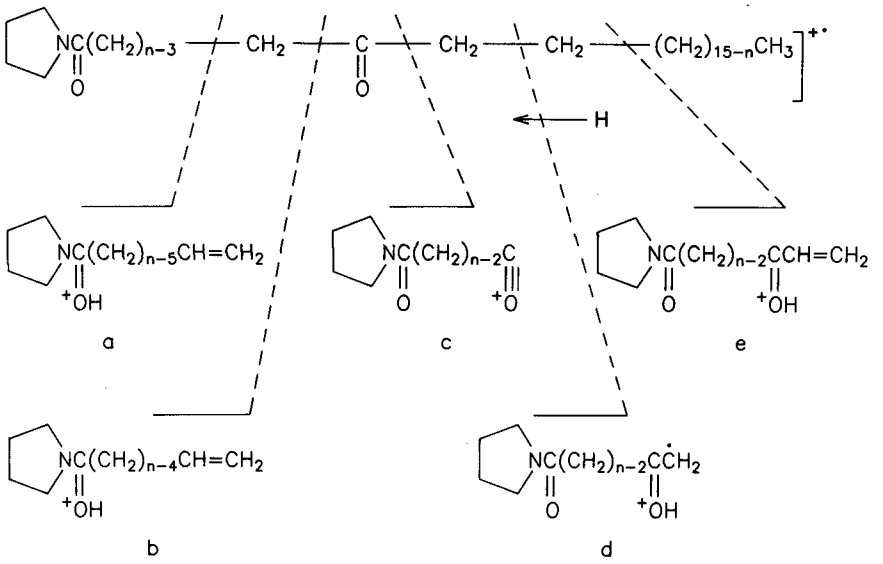

FIG. 1. Proposed fragmentation modes in mass spectra of pyrrolidides of oxooctadecanoic acids; n is position of substituent.

TABLE 1

Characteristic Ions in EI Mass Spectra of Pyrrolidides of the 16 Isomeric Oxooctadecanoic Acids

Position of oxo carbon n	Ion m/z and relative intensities of ions: Ion type[p] a	b	c	d	e	$[M]^+$ m/z 351	Base peak
2[q]	–	98 (100)	126 (3)	141 (1)	154 (1)	(5)	98
3[r]	98 (25)	112 (89)	140 (11)	155 (95)	168 (31)	(6)	70
4[s]	112 (5)	126 (31)	154 (100)	169 (50)	182 (17)	(5)	154
5	126 (16)	140 (32)	168 (27)	183 (34)	196 (22)	(5)	113
6	140 (21)	154 (26)	182 (7)	197 (16)	210 (12)	(4)	113
7	154 (45)	168 (45)	196 (11)	211 (8)	224 (12)	(6)	113
8	168 (69)	182 (28)	210 (13)	225 (3)	238 (12)	(8)	113
9	182 (62)	196 (18)	224 (9)	239 (2)	252 (7)	(5)	113
10	196 (67)	210 (16)	238 (7)	253 (2)	266 (6)	(5)	113
11	210 (47)	224 (10)	252 (6)	267 (1)	280 (5)	(3)	113
12	224 (26)	238 (4)	266 (4)	281 (1)	294 (3)	(2)	113
13	238 (46)	252 (7)	280 (7)	295 (1)	308 (4)	(3)	113
14	252 (40)	266 (5)	294 (7)	309 (1)	322 (3)	(3)	113
15	266 (21)	280 (3)	308 (5)	–	–	(2)	113
16	280 (21)	294 (3)	322 (5)	–	–	(2)	113
17	294 (28)	308 (3)	336 (1)	–	–	(3)	113

[p]Ions have structures shown in Fig. 1 except when n=2 to 4 ion is $(CH_2\text{-}CH_2)_2NC\dot{O}(CH_2)^{\bullet}_{n-3}$ and when n=2 or 3 ion b is $(CH_2\text{-}CH_2)_2NC\dot{O}(CH_2)^{\bullet}_{n-2}$.

[q]Other ions are: 113 (7), 253 (5).

[r]Other ions are: 113 (51).

[s]Other ions are: 113 (7), 127 (28).

complete series of isomers. The base peak in spectra of the 5- to 17-oxo derivatives is ion m/z 113, which was mentioned above as the base peak in the spectra of nonoxygenated pyrrolidides. The five ions a to e (Fig. 1 and Table 1) appear in spectra of all the oxo pyrrolidides ex-

cept the last three. The most prominent ion is ion a, formed by cleavage between carbons α- and β- to the oxo group on the pyrrolidide side. This type of cleavage was noted previously in spectra of pyrrolidides of oxo acids (2,13) and also has been noted in spectra of other oxo compounds with a second ionizable center also containing an oxo group, such as oxo β-diketones (21). This ion presumably is formed by ADF in which a radical site (leading to cleavage) is produced at a carbon remote from the amide group by migration of a hydrogen to the amide carbonyl (12); the ADF effect is less directed when the oxo group is far from the amide. The actual hydrogen abstraction site is, however, substituent directed and is probably the carbon γ- to the carbonyl on the pyrrolidide side (12). In contrast, hydrogen abstraction is much less specific in the spectrum of the pyrrolidide of octadecanoic acid (12,13). Fragment a is most intense in spectra of isomers with the oxo group near the center of the chain, that is, of 7- to 14- oxo derivatives.

Ion b, from α-cleavage on the pyrrolidide side of the carbonyl group, is prominent only in spectra of 5- to 8-oxo pyrrolidides and is weak in spectra of those with more remote oxo groups. It also is formed by ADF with abstraction from the β-carbon on the pyrrolidide side of the carbonyl. Ions type c, d and e correspond to cleavage on the other side of the oxo group and are weak in all spectra except those of 3- to 5-oxo pyrrolidides. Ion d is the McLafferty rearrangement ion and contains a hydrogen from the γ-carbon (15). The molecular ion is weak (2-8%) in all spectra, particularly in those with the oxo group remote from the pyrrolidide group. A further indication of the position of the oxo carbonyl is given by a conspicuous gap in the series of acyl pyrrolidide ions (of which ions a and b are members). The number of carbons in the acyl chain of the missing, or extremely weak, ion in the series is the number of the oxo carbon. Thus, in the spectrum of 9-oxo pyrrolidide the C_9 acyl pyrrolidide ion, m/z 210, is very weak.

In contrast to these relatively simple spectra, those of methyl oxooctadecanoates showed a much larger number of fragments, as cleavages on both sides of the carbonyl group were much more intense. Ions analogous to ions a, b, c and d were all quite prominent in spectra of methyl 7-, 8- and 9-oxooctadecanoates and, as mentioned above, they generally were accompanied by ions lower in mass by 32 amu (1). In addition, α-cleavage on the ester side and McLafferty rearrangement ions from cleavage on this side were important ions. Cleavage on the ester side of the carbonyl occurs to only a very minor extent in fragmentation of oxo pyrrolidides and in fact is significant only in the spectrum of 2-oxo pyrrolidide where ion m/z 253 [$\overset{+}{C}O(CH_2)_{15}CH_3$] forms 5% of the base peak.

Due to the proximity of the carbonyl groups, spectra of 2-, 3- and 4-oxo pyrrolidides are considerably different from those of the other isomers. In the spectrum of 2-oxo pyrrolidide, ion b from cleavage between the carbonyls with the charge retained by the amide is the base peak with m/z 98. On the other hand, in the spectrum of methyl 2-oxooctadecanoate, the base peak is ion m/z 253 [$(M-59)^+$] with charge retained by the oxo carbonyl (1). Ions b and d are very strong in the spectrum of 3-oxo pyrrolidide and indicate the structure, but the spectrum of methyl 3-oxooctadecanoate does not contain any corresponding characteristic ions (1). Ion c is the base peak in the spectrum of 4-oxopyrrolidide; ion d is also prominent, but in the spectrum of methyl 4-oxooctadecanoate the base peak is the McLafferty rearrangement ion analogous to ion d (1).

Examination of spectra of deuterium-labelled derivatives confirmed the formation of ions a to e (Fig. 1). In the spectrum of the pyrrolidide of 11-oxo[8-$^2H_2$]octadecanoic acid, ions a to d all increased by 2 amu and in the spectrum 11-oxo[14-$^2H_2$]octadecanoic acid pyrrolidide ion d increased by 1 amu, showing that it was formed by the McLafferty rearrangement with transfer of deuterium from C-14. Masses of the other ions were unchanged. There was no change in ions a to d in the spectrum of the pyrrolidide of 13-oxo[15-$^2H_2$]octadecanoic acid, but ion e increased by 2 amu.

Mass Spectra of Pyrrolidides of Hydroxy Octadecanoic Acids

The useful fragmentation is shown in Figure 2, and the intensities are given in Table 2. Spectra of the 5- to 18-hydroxy pyrrolidides will be considered before those of the 2- to 4-hydroxy derivatives. Ions a and b, which are important in spectra of oxo pyrrolidides, again appear in those of hydroxy pyrrolidides, but intensities are considerably lower. Thus, ions a and b in the spectrum of 9-oxo pyrrolidide are 62% and 18%, but in the spectrum of 9-hydroxy pyrrolidide they are only 12% and 5%.

The secondary alcohol group is another fragmentation site which competes more effectively with the pyrrolidide site than did the oxo group; SDF is now stronger compared to ADF. Ion g is the most important diagnostic ion, with an intensity ranging from 20% to 45% in spectra of 5-hydroxy to 14-hydroxy pyrrolidides, and results from α-cleavage at the hydroxylated carbon on the terminal methyl side. This type of

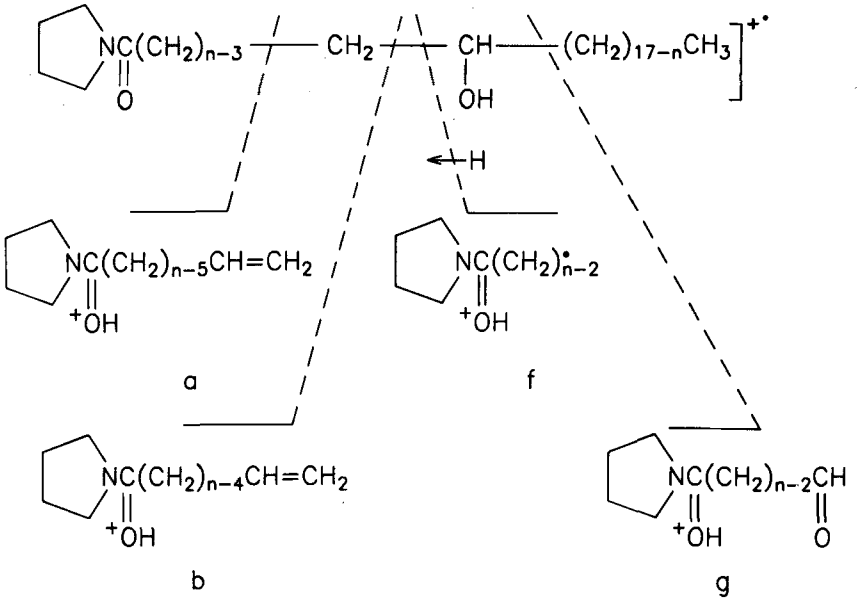

FIG. 2. Proposed fragmentation modes in mass spectra of pyrrolidides of hydroxy octadecanoic acids; n is position of substituent.

TABLE 2

Characteristic Ions in EI Mass Spectra of Pyrrolidides of the 17 Isomeric Hydroxyoctadecanoic Acids

Hydroxy group position n	Ion m/z and relative intensities of ions						
	Ion type[p]						
	a	b	f	g	$[M-18]^+$ m/z 335	$[M]^+$ m/z 353	Base peak
2[q]	–	98 (81)	99 (88)	128 (29)	(2)	(5)	129
3	98 (67)	112 (10)	113 (83)	142 (100)	(5)	(2)	142
4	112 (4)	126 (11)	127 (16)	156 (46)	(1)	(0.2)	113
5[r]	126 (13)	140 (11)	141 (11)	170 (37)	(5)	(0.3)	113
6	140 (5)	154 (8)	155 (5)	184 (29)	(3)	(0.4)	113
7	154 (5)	168 (7)	169 (4)	198 (20)	(1)	(1)	113
8	168 (12)	182 (5)	183 (6)	212 (34)	(2)	(1)	113
9	182 (12)	196 (5)	197 (5)	226 (45)	(4)	(1)	113
10	196 (10)	210 (3)	211 (4)	240 (43)	(3)	(1)	113
11	210 (9)	224 (3)	225 (4)	254 (40)	(3)	(1)	113
12	224 (8)	238 (2)	239 (3)	268 (35)	(3)	(1)	113
13	238 (10)	252 (3)	253 (3)	282 (37)	(4)	(1)	113
14	252 (5)	266 (1)	267 (1)	296 (23)	(3)	(1)	113
15	266 (5)	280 (1)	281 (1)	310 (12)	(2)	(1)	113
16	280 (5)	294 (1)	295 (1)	324 (10)	(2)	(1)	113
17	294 (5)	308 (1)	309 (1)	338 (2)	(2)	(1)	113
18	–	–	–	–	(0.2)	(0.5)	113

[p]Ions have structures shown in Fig. 2 except when n=2 to 4 ion a is $(CH_2\text{-}CH_2)_2NC\overset{+}{O}(CH_2)^{\bullet}_{n-3}$ and when n=2 or 3 ion b is $(CH_2\text{-}CH_2)_2NC\overset{+}{O}(CH_2)^{\bullet}_{n-2}$.

[q]Other ions were: 100 (90), 142 (9), 171 (7).

[r]Other ions are: 127 (12), 166 (11).

ion is an important one in spectra of secondary alcohols (22) and methyl hydroxy octadecanoates, which also contain ions formed from it by loss of 32 amu (1). The other characteristic ion is ion f, produced by cleavage of the same bond as in formation of b but accompanied by migration of a hydrogen. It appears in all the spectra of hydroxy pyrrolidides and intensities decrease steadily from 11% in the spectrum of 5-hydroxy pyrrolidide to 1% in that of 17-hydroxy pyrrolidide. Analogous ions apparently are much more intense in spectra of methyl hydroxy esters and, in fact, one is the base peak in the spectrum of methyl 17-hydroxyoctadecanoate (1). It has been shown to be formed by transfer of the hydroxyl hydrogen to the carbonyl oxygen (23).

When the hydroxyl is near the terminal methyl group, intensities of all the ions due to SDF are weak, particularly in the spectrum of 17-hydroxy pyrrolidide, though they are adequate for structure determination. The spectrum of 18-hydroxy pyrrolidide contained no characteristic ions and was very similar to that of the pyrrolidide of octadecanoic acid (8,12,13) except for the very weak $[M]^+$ and $[M-18]^+$ ions.

Ion g is also a major ion (46% of the base peak m/z 113) in the spectrum of 4-hydroxy pyrrolidide, and this type of cleavage gave the base peak (117-32) in the spectrum of methyl 4-hydroxyoctadecanoate (1). It is the base peak in the spectrum of 3-hydroxyoctadecanoate (1). Ions a and f are also very strong in this pyrrolidide spectrum (Table 2). In the spectrum of 2-hydroxy pyrrolidide, ion f is strong but the base peak is ion m/z 129, which is the McLafferty rearrangement ion $(CH_2\text{-}CH_2)_2NC(\dot{O}H)\dot{C}HOH)$. This type of cleavage is less pronounced in the spectrum of methyl 2-hydroxyoctadecanoate (1). $[M]^+$ is only 1% or less in spectra of hydroxy pyrrolidides, but $[M\text{-}H_2O]^+$ ions are slightly stronger, generally 2-5%.

Spectra of specifically deuterated pyrrolidides again confirmed the proposed fragmentations. As shown in Table 3, the ions a, b, f and g all increased by 2 amu in spectra of pyrrolidides of 8-hydroxy[2-$^2H_2$]-, 8-hydroxy[5-$^2H_2$]-, 11-hydroxy[8-$^2H_2$]- and 11-hydroxy [10,12-$^2H_4$]octadecanoic acids, but only $[M-18]^+$ and M^+ increased by 2 amu in spectra of pyrrolidides of 11-hydroxy[14-$^2H_2$]- and 13-hydroxy[15-$^2H_2$]octadecanoic acids. Also in the spectrum of 8-hydroxy[2-$^2H_2$]pyrrolidide, the base peak appeared at m/z 115 (from m/z 113) in agreement with previous conclusions about the formation of this McLafferty ion (9). In the spectrum of the pyrrolidide of 17-hydroxy[17-^2H]octadecanoic acid, ion g, the $[M-18]^+$ ion and the M^+ ion increased by 1 amu. In the spectrum of the pyrrolidide of 17-hydroxy[16,18-$^2H_5$]octadecanoic acid, ions b, f and g increased by 2 amu and the $[M-18]^+$ and M^+ ions by 5 amu.

Mass Spectra of TMS Ethers of Pyrrolidides of Hydroxy Octadecanoic Acids

Fragmentation of TMS ethers of hydroxy pyrrolidides is shown in Figure 3, and intensities of the ions are listed in Table 4. The TMS

TABLE 3

Comparison of Ions in EI Mass Spectra of Pyrrolidides of Undeuterated and Specifically Deuterated Hydroxyoctadecanoic Acids

Hydroxyl group position n	Deuterium position	Ion m/z and relative intensities of ions: Ion type[p] a	b	f	g	$[M-18]^+$	$[M]^+$	Base peak
8	–	168 (12)	182 (5)	183 (6)	212 (34)	335 (2)	353 (1)	113
8	2,2	170 (12)	184 (6)	185 (9)	214 (56)	337 (3)	355 (2)	115
8	5,5	170 (7)	184 (7)	185 (11)	214 (46)	337 (4)	355 (3)	113
11	–	210 (9)	224 (3)	225 (4)	254 (40)	335 (3)	353 (1)	113
11	8,8	212 (4)	226 (4)	227 (5)	256 (55)	337 (5)	355 (2)	113
11	10,10,12,12	210 (7)	226 (2)	227 (2)	256 (15)	338 (2) 339 (1)	357 (0.5)	113
11	14,14	210 (9)	224 (6)	225 (10)	254 (65)	337 (9)	355 (3)	113
13	–	238 (10)	252 (3)	253 (3)	282 (37)	335 (4)	353 (1)	113
13	15,15	238 (5)	252 (1)	253 (1)	282 (18)	337 (2)	355 (1)	113
17	–	294 (5)	308 (1)	309 (1)	338 (2)	335 (2)	353 (1)	113
17	17	294 (8)	308 (3)	309 (3)	339 (7)	336 (4)	354 (2)	113
17	16,16,18,18,18	294 (6)	310 (2)	311 (2)	340 (4)	340 (4)	358 (2)	113

[p]Ions have structures shown in Fig. 2.

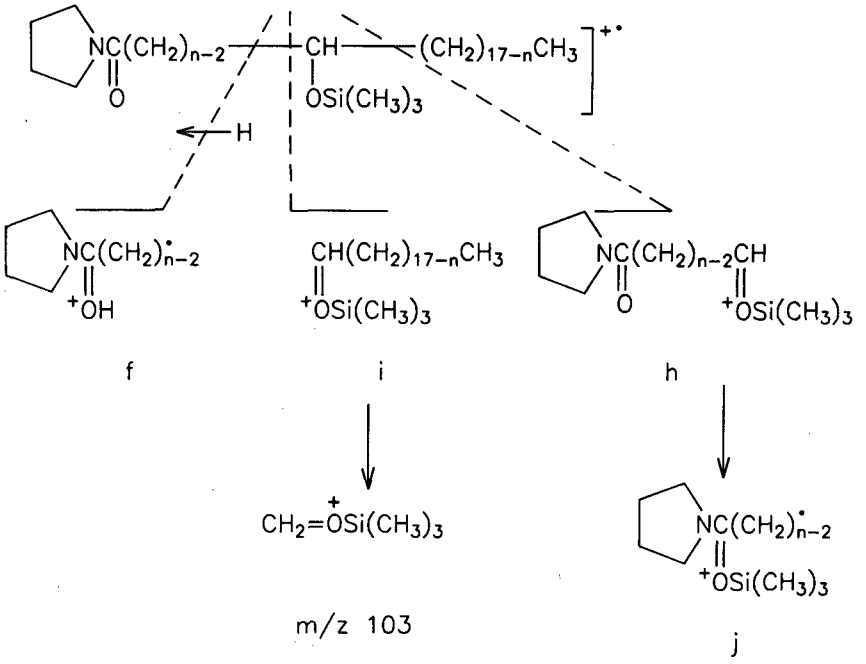

FIG. 3. Proposed fragmentation modes in mass spectra of TMS ethers of pyrrolidides of hydroxy octadecanoic acids; n is position of substituent.

ethers have two nearly equivalent centers where ionization and fragmentation can occur. In spectra of oxo and hydroxy pyrrolidides ADF yielded ions a and b, but these are absent from those of the TMS ethers. SDF gives ion i, which contains the terminal methyl part of the chain and is formed by α-cleavage on the amide side of the oxygenated carbon with the charge remaining on the silyl oxygen. Fragment i is also prominent in spectra of TMS ethers of secondary alcohols (22) and methyl hydroxy esters (7). Ion i is more intense in spectra of 10- to 17-trimethylsilyloxy pyrrolidides (Table 4), indicating that SDF occurs more readily when the oxygenated carbon is well separated from the amide group.

Ion h, the other expected ion, results from α-cleavage on the methyl terminal side of the oxygenated carbon. This ion is approximately as intense as ion m/z 113 in spectra of the 5- to 11-TMS oxy pyrrolidides, but less strong, though still a major ion in spectra of 12- to 16-TMS derivatives, where the TMS group is further from the amide.

Ion f was unexpected in spectra of the TMS derivatives because hydrogen migration had occurred. In spectra of hydroxy pyrrolidides, as in those of hydroxy methyl esters (23), this ion is formed by transfer of the hydroxyl hydrogen, but this source is not present in TMS ethers. The origin of the hydrogen in ion f was determined using deuterated derivatives and is discussed below. Ion f is 2-3 times as intense in spectra of TMS pyrrolidides as it is in spectra of hydroxy pyrrolidides, indicating that it is more readily formed.

The fourth ion j results from transfer of the TMS group to the amide carbonyl followed by a second α-cleavage on the amide side of the oxygenated carbon, or transfer of TMS may precede any cleavage as was postulated when this type of migration was first noted in spectra of TMS ethers of hydroxy methyl esters (5). Ion j is rather weak (1-3%) in most spectra listed in Table 4 except when the substituent is close to the amide.

As in the spectra of the oxo and hydroxy pyrrolidides, those of the 2-, 3- and 4-TMS ethers have special characteristics. In the spectrum of the 4-TMS ether, h is the major diagnostic ion and ion i is very weak. Ion m/z 313, however, is 21% of the base peak; presumably it is $\dot{C}H_2CH(\dot{O}TMS)(CH_2)_{13}CH_3$ resulting from cleavage of the 2,3 bond. There is no analogous ion in the spectrum of the TMS ether of methyl 4-hydroxy esters. The 2,3 bond is also cleaved in formation of the base peak m/z 113. A relatively intense ion m/z 185 (59%) probably also

TABLE 4

Characteristic Ions in EI Mass Spectra of Pyrrolidides of Trimethylsilyl Ethers of the 17 Isomeric Hydroxyoctadecanoic Acids

	Ion m/z and relative intensities of ions[p]							
	Ion type							
TMS group position n	f	h	i	j	$[M-90]^+$ m/z 335	$[M-15]^+$ m/z 410	[M] m/z 425	Base peak
2[q]	–	–	327 (46)	171 (100)	–	(12)	–	171
3[r]	113 (59)	214 (100)	313 (1)	185 (13)	(8)	(40)	(4)	214
4[s]	127 (12)	228 (64)	299 (3)	199 (11)	(3)	(17)	(2)	113
5	141 (19)	242 (100)	285 (9)	213 (7)	(15)	(16)	(2)	113,242
6	155 (16)	256 (98)	271 (13)	227 (3)	(9)	(20)	(2)	113
7[t]	169 (16)	270 (100)	257 (15)	241 (3)	(6)	(16)	(2)	270
8	183 (14)	284 (96)	243 (13)	255 (2)	(5)	(11)	(1)	113
9[u]	197 (6)	298 (100)	229 (13)	269 (1)	(6)	(13)	(1)	298
10	211 (13)	312 (97)	215 (31)	283 (2)	(10)	(18)	(2)	113
11[v]	225 (9)	326 (100)	201 (25)	297 (2)	(5)	(12)	(2)	326
12	239 (12)	340 (80)	187 (47)	311 (3)	(5)	(17)	(3)	113
13	253 (9)	354 (69)	173 (52)	325 (2)	(5)	(17)	(3)	113
14	267 (8)	368 (70)	159 (56)	339 (2)	(4)	(17)	(3)	113
15	281 (7)	382 (61)	145 (63)	353 (2)	(4)	(16)	(3)	113
16	295 (6)	396 (55)	131 (60)	367 (1)	(3)	(17)	(3)	113
17[w]	309 (9)	410 (26)	117 (66)	381 (1)	(3)	(26)	(3)	113
18	–	–	103 (6)	–	(2)	(41)	(3)	113

[p]Ions have structures shown in Fig. 3.
[q]Other ions are: 103 (30), 201 (11).
[r]Other ions are: 152 (21), 228 (28).
[s]Other ions are: 185 (59), 313 (21).
[t]Other ions are: 113 (82).
[u]Other ions are: 113 (48).
[v]Other ions are: 113 (89).
[w]See discussion of ion m/z 410 in text.

is formed by cleavage of this 2,3 bond, but because this ion is also present, though weaker, in all the other TMS spectra its mode of formation is discussed later.

Ion h is the base peak in the spectrum of the 3-TMS ether and corresponds to ion m/z 175, which is a major ion in the spectrum of the TMS ether of the methyl ester of 3-hydroxy acids (4,7). It also corresponds to ion g, also the base peak, in the spectrum of 3-hydroxy pyrrolidide (see above). The base peak in the spectrum of the 2-TMS ether is ion j, which may be formed from the McLafferty ion, m/z 201 (11%), by elimination of formaldehyde, or directly from the molecular ion (5). Ion i was the other major ion in this spectrum and also appears in that of the TMS ether of methyl esters of 2-hydroxy acids (4,7).

Another important ion is the $[M-15]^+$ ion, usually prominent in spectra of silyl ethers, formed by loss of a silyl methyl group (24). The intensity ranges from 11 to 41%, higher than that observed for $[M-15]^+$ in spectra of TMS ethers of most hydroxy methyl esters (4,7). The $[M-15]^+$ ions in spectra of TMS ethers of pyrrolidides of cutin acids were markedly less intense (2). The $[M-90]^+$ ion (loss of TMSiOH) is a weak ion in most spectra. M^+ is also weak but is discernible in all spectra; in contrast, M^+ was not detected in spectra of most TMS ethers of methyl esters of hydroxyhexadecanoic acids (7).

Spectra of TMS ethers of 17- and 18-hydroxy pyrrolidides also differ from the rest. In the spectrum of the 17-TMS ether the $[M-15]^+$ ion and ion h have the same mass, 410 amu, but as shown below using deuterated compounds, $[M-15]^+$ contributes most to ion m/z 410. In the spectrum of the 18-TMS ether the only diagnostic ion is ion i, m/z 103, but $[M-15]^+$ is more than twice as intense as it is in spectra of all the other ethers except that of the 3-TMS ether. The TMS ethers of primary alcohols usually have much more intense $[M-15]^+$ ions than do ethers of secondary alcohols (22).

Mass Spectra of Deuterated TMS Ethers of Pyrrolidides of Hydroxy Octadecanoic Acids

Fragmentation shown in Figure 3 was supported by spectra of TMS ethers of pyrrolidides of selectively deuterated hydroxy octadecanoic

acids (Table 5). Ions f, h and j increased by 2 amu in spectra of TMS ethers of pyrrolidides of 8-hydroxy[2-$^2H_2$]-, 8-hydroxy[5-$^2H_2$]- and 11-hydroxy[8-$^2H_2$]octadecanoic acids. Ion i increased by 2 amu in spectra of TMS ethers of pyrrolidides of 11-hydroxy[10,12-$^2H_4$]- (ions h and j also increased by 2 amu in this spectrum) and 13-hydroxy[15-$^2H_2$]octadecanoic acids. Ions h and i increased by 1 amu in the spectrum of the TMS ether or the pyrrolidide of 17-hydroxy[17-^2H]octadecanoic acid.

In the spectrum of the TMS ether of the pyrrolidide of 17-hydroxy[16,18-$^2H_5$]octadecanoic acid, ions h and j increased by 2 amu and ion i by 3 amu. In this spectrum the $[M-15]^+$ ion, which increases by 5 amu to 415, is about four times as intense as ion h, which only increases to 412 amu. This spectrum and that of the TMS ether of the pyrrolidide of 11-hydroxy[10,12-$^2H_4$]octadecanoic acid also provide an explanation of the formation of ion f. Ion f increased by 3 amu, indicating that deuterium is transferred from C-18 in the former compound and from C-12 in the latter to the amide carbonyl. The mechanism of formation of ion f is shown in Figure 4. Hydrogen is abstracted by the amide carbonyl from C-(n+1), and the radical site at this carbon initiates α-cleavage on the other side of the oxygenated carbon. This long range migration is another example of ADF. Ion f was unchanged in spectra of the TMS ethers of 13-hydroxy[15-$^2H_2$]- and 11-hydroxy-[14-$^2H_2$]octadecanoic acids, showing that there is no abstraction from the β- or γ-carbons. There was no ion corresponding to ion f in spectra of TMS ethers of methyl hydroxyhexadecanoates (7), indicating the strength of ADF in pyrrolidides. Also, ion f is still of moderate (9%) intensity even in the spectrum of the TMS ether of 17-hydroxy pyrrolidide (Table 4); in spectra of the hydroxy pyrrolidides it is very weak when the hydroxyl is near the end of the chain (Table 2).

Table 5 also compares ions in spectra of TMS ethers of 2-, 4- and 17-hydroxy pyrrolidides with those in the spectra of the corresponding tri[$^2H_3$]methylsilyl ethers. In the spectrum of this derivative of the 2-hydroxy pyrrolidide, ions i, j and ion m/z 201 all increased by 9 amu, showing that these ions, and ion j in particular, have the proposed structures and in the case of ion j are not formed in some other way by loss of silyl methyl groups. In the spectrum of the deuterated TMS ether of 4-hydroxy pyrrolidide, ions h, i and j and ion m/z 313 all increased by 9 amu as expected. Ions h, i and j also increased by 9 amu in the spectrum of this derivative of 17-hydroxy pyrrolidide and ion h, m/z 419, can be distinguished from the $[M-CD_3]^+$ ion with m/z 416.

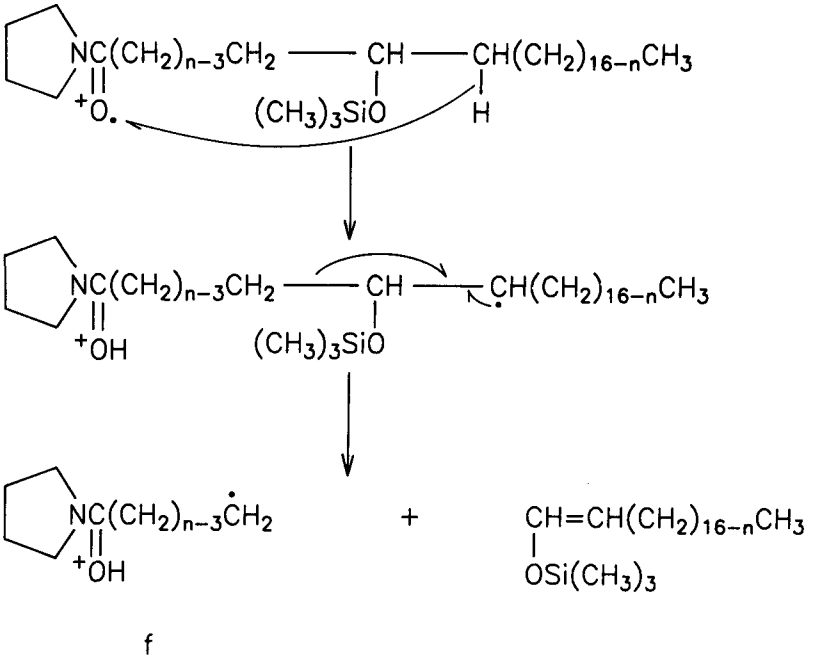

FIG. 4. Formation of ion f in fragmentation of TMS ether of pyrrolidide of n-hydroxyoctadecanoic acid.

TABLE 5

Comparison of Ions in EI Mass Spectra of Undeuterated and Specifically Deuterated TMS Ethers of Pyrrolidides of Hydroxyoctadecanoic Acids

TMS group position n	Deuterium position	Ion m/z and relative intensities of ions[p]							
		Ion type							
		f	h	i	j	$[M-90]^+$	$[M-15]^+$	$[M]^+$	Base peak
2[q]	–	–	–	327 (46)	171 (100)	–	410 (12)	–	171
2[r]	TMS	–	–	336 (42)	180 (86)	–	416 (8)	434 (1)	43
4[s]	–	127 (12)	228 (64)	299 (3)	199 (11)	335 (3)	410 (17)	425 (2)	113
4[t]	TMS	127 (9)	237 (51)	308 (3)	208 (9)	335 (3)	416 (13)	434 (2)	113
8	–	183 (14)	284 (96)	243 (13)	255 (2)	335 (5)	410 (11)	425 (1)	113
8	2,2	185 (19)	286 (100)	243 (17)	257 (3)	337 (2)	412 (18)	427 (2)	286
8[u]	5,5	185 (51)	286 (100)	243 (25)	257 (4)	337 (9)	412 (20)	427 (2)	286
11	–	225 (9)	326 (100)	201 (25)	297 (2)	335 (5)	410 (12)	425 (2)	326
11	8,8	227 (15)	328 (100)	201 (40)	299 (3)	337 (8)	412 (17)	427 (3)	328
11	10,10,12,12	228 (10)	328 (89)	203 (40)	299 (2)	338 (2) 339 (3)	414 (14)	429 (2)	113
11	14,14	225 (15)	326 (100)	203 (39)	297 (3)	337 (8)	412 (20)	427 (3)	326
13	–	253 (9)	354 (69)	173 (52)	325 (2)	335 (5)	410 (17)	425 (3)	113
13	15,15	253 (9)	354 (69)	175 (53)	325 (2)	337 (4)	412 (11)	427 (1)	113
17	–	309 (9)	410 (26)	117 (66)	381 (1)	335 (3)	410 (26)	425 (3)	113
17	17	309 (9)	411 (33)	118 (42)	381 (1)	336 (2)	411 (33)	426 (4)	113
17	16,16,18,18,18	312 (7)	412 (5)	120 (61)	383 (1)	338 (3) 339 (1) 340 (1)	415 (20)	430 (3)	113
17	TMS	309 (12)	419 (7)	126(100)	390 (1)	335 (3)	416 (18)	434 (4)	126

[p]Ions have structures shown in Fig. 3.

[q]Other ions were: 103 (30), 201 (11).

[r]Other ions are: 112 (14), 210 (6).

[s]Other ions are: 185 (59), 313 (21).

[t]Other ions are: 194 (43), 322 (17).

[u]Ion f is more intense because it coincides with ion m/z 185 $[(CH_2\text{-}CH_2)_2NC(\dot{C}H_2)=\dot{O}Si(CH_3)_3]$.

Finally there also are several ions which appear in most spectra of the TMS ethers; one of these is ion m/z 103 which has a relative intensity of about 7 to 12% in spectra of TMS ethers of 5- to 15-hydroxy pyrrolidides. It is most probably derived from ion i because it is also present in spectra of TMS ethers of secondary alkanols when the oxygenated carbon is separated from the terminal methyl by at least 2 carbons (22) and in spectra of TMS ethers of comparable hydroxy methyl esters (4,7,25). This ion is more intense (30%) in the spectrum of the TMS ether of 2-hydroxy pyrrolidide presumably because ion i forms 46% of the base peak (Table 4). Ion m/z 103 is characteristic of spectra of TMS ethers of primary alkanols (22) and has the structure $CH_2=\dot{O}Si(CH_3)_3$ (24). It most probably has the same structure in spectra of TMS ethers of secondary alcohols because it increases to m/z 112 in spectra of tri[$^2H_3$]-methylsilyl ethers (Table 5). The formation of ion m/z 103 requires transfer of hydrogen from a neighboring carbon. The result of deuterium labelling is shown in Table 6 and indicates that deuterium is transferred to an extent of about 50% from the α-carbon, 24% from the β-carbon and 8% from the γ-carbon; the rest presumably comes from other carbons. The radical site then initiates α cleavage yielding ion m/z 103 and an olefin which has, preferably, three or more carbons.

TABLE 6

Effect of Deuterium Labeling on Ions m/z 103 and 104 in Mass Spectra of TMS Ethers of Pyrrolidides of Hydroxyoctadecanoic Acids

TMS group position	Deuterium position	Ion m/z and intensity[a]	
		103	104
11	–	99	1
11	10,10,12,12	49	51
13	15,15	76	24
11	14,14	92	8

[a]Calculated from multiple ion detection for ions m/z 103 and 104 by subtraction of C, H and Si isotope contributions of ion m/z 103 to ion m/z 104 (15).

Two other peaks, m/z 185 and 198, appear in spectra of all the TMS pyrrolidides and most probably have the structures shown in Figure 5, analogous to ions m/z 146 and 159 in spectra of TMS ethers of methyl hydroxy esters (5). It was proposed that transfer of the TMS group to the carbonyl is followed by hydrogen abstraction, by the alkoxy radical, from either C-4 or C-2. In the former case fragmentation yielded ion m/z 146 and in the latter yielded ion m/z 159; it was pointed out that these rearrangements are the TMS analogs of formation of ions m/z 74 (McLafferty ion) and 87 in spectra of methyl alkanoates (5). As was mentioned above (and Table 4), ion m/z 185 is quite intense in the spectrum of the TMS ether of 4-hydroxy pyrrolidide, and the special case of its formation is proposed in Figure 6. Ions 185 and 198 are weak in the spectrum of the 2-TMS derivative, but in those of the 3- to 11-TMS compounds ion m/z 185 ranges from 13 to 41% (except for that of the 4-TMS compound above) and ion m/z 198 from 2 to 21%; in the 12- to 18-TMS derivatives the former ion ranges from 38 to 50% and the latter from 8 to 17%. In spectra of TMS ethers of hydroxy methyl esters, on the other hand, ions m/z 146 and 159 are much weaker, rarely exceeding 5-10%; ion m/z 159 is the more intense (7). Ion m/z 185 also was present in spectra of TMS ethers of pyrrolidides of cutin acids (2). This ion increased mainly by two amu and ion m/z 198 by one amu in the spectrum of the TMS ether of the pyrrolidide of 8-hydroxy[2-H_2]octadecanoic acid and, as expected, both increased by nine amu in spectra of tri[$^2H_3$]methylsilyl derivatives.

CONCLUSIONS

The preceding discussion of the mass spectra of the pyrrolidides of oxygenated octadecanoic acids shows that they are feasible alternative derivatives to methyl esters. Spectra of the oxo and hydroxy pyrrolidides indicate the position of the substituents in all isomers by one major fragment and confirm it with four other ions for oxo compounds and three others for hydroxy compounds. Ions resulting from further breakdown of the diagnostic ion, with consequent decrease in intensity as occurs in spectra of methyl esters by loss of 32 amu (1), are not formed. It has been noted that mass spectra of other oxygenated pyrrolidides do not contain ions characteristic of the terminal part of the chain (16). This is not a serious disadvantage in the present investigation, because chain length is shown by M^+ (and $[M-18]^+$ in hydroxy compounds); besides, spectra of hydroxy methyl esters do not yield ions containing this part of the chain, either (1). Structural variation in this part probably would be indicated by changes in other ions in the spectrum (for oxo compounds at least) as is seen in spectra of pyrrolidides of alkyl substituted alkanoic acids (13,26).

Mass spectra of TMS ethers of pyrrolidides are more informative than those of hydroxy pyrrolidides because they contain ion i, characteristic of the methyl end of the chain. Ion h is more intense in spectra of TMS ethers of pyrrolidides than the corresponding ion in those of the ethers of hydroxy methyl esters, but ion i is more intense in the latter spectra (7). This substituent position is indicated by spectra of

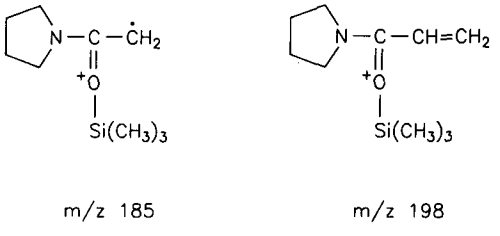

FIG. 5. Structures of ions m/z 185 and 198 which appear in mass spectra of TMS ethers of pyrrolidides of hydroxy octadecanoic acids.

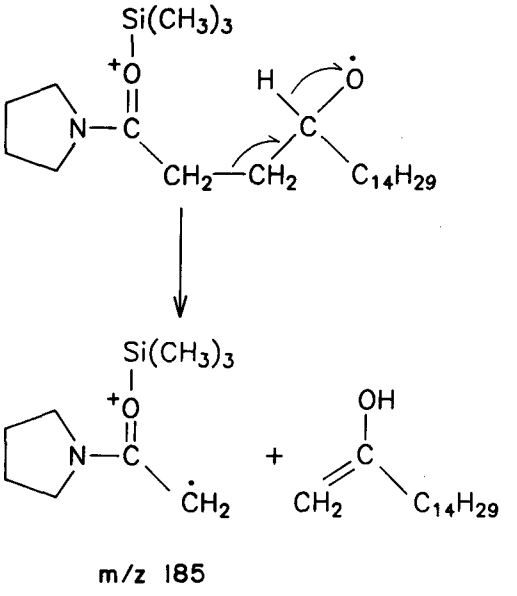

FIG. 6. Formation of ion m/z 185 in fragmentation of TMS ether of pyrrolidide of 4-hydroxy octadecanoic acid.

TMS ethers of both hydroxy pyrrolidides and hydroxy methyl esters. Pyrrolidides are, however, preferred as derivatives of 4- and 5-hydroxy acids because they are easily formed and more stable than the methyl esters which readily lactonize (7,17). Also, intensity of the $[M-15]^+$ ion, which indicates overall chain length, is much greater in the spectra of TMS ethers of pyrrolidides than it is in those of TMS ethers of most hydroxy methyl esters (7).

In previous studies of mass spectra of pyrrolidides of long chain acids rules were formulated for determining position of methyl substituents (26) and of double and triple bonds (10-12) from the m/z and intensities of the ions; such rules could be applied in computerized examination of spectra. They refer to changes in the series of weak ions, corresponding to the C_n acyl pyrrolidide fragments beginning at m/z 126 (C_3) and increasing by intervals of 14 amu, which appears in the spectrum of the pyrrolidide of octadecanoic acid (8,12,13). Thus, for spectra of oxo pyrrolidides the rule would be that a strong ion at C_{n-2} (ion a) and a very weak one at C_n indicates carbonyl oxygen at C_n. In the spectra of hydroxy pyrrolidides the series of acyl fragments is missing beyond ion g, but a rule may be stated that a very weak ion at C_n and a strong ion at m/z for $C_{n+1}+2$ (ion g) indicates hydroxyl at C_n. Such a rule determines substitution at C-3 to C-17. Most of the acyl fragments are very weak in spectra of TMS ethers of pyrrolidides so that a simple rule cannot be drawn up. In most isomers, however, substituent position can be determined from the major even mass ion (h) and confirmed by a generally strong odd mass ion (i).

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Chylomicron Remnant and Asialoglycoprotein Metabolism are Independent

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ABSTRACT

Because of the considerable similarities between the hepatic metabolism of chylomicron remnants and asialoglycoproteins, the hypothesis that they might share a cell surface receptor or a common step in internalization was tested. Unlabeled chylomicron remnants did not reduce the binding of ^{125}I-asialoglycoprotein to plasma membranes, but did compete for ^{125}I-chylomicron remnant binding. The converse also was true. This suggested the receptors were distinct. The two substances did not compete with each other for removal by the isolated perfused rat liver. This suggests that no potentially common post binding events can become rate limiting.

In conclusion, despite similarities in their removal and metabolism, chylomicron remnants and asialoglycoproteins are metabolized independently.

Lipids 20:664-667, 1985.

INTRODUCTION

One important hepatic function is the removal of macromolecules from the blood. Details of the removal mechanisms are known for some, but not all, transported substances. One of the most thoroughly studied transport systems is that by which desialated proteins are removed from the circulation and degraded by the liver. It is established that the hepatic removal of proteins lacking a terminal sialic acid and containing a terminal galactose proceeds by a pathway known as receptor mediated endocytosis (1). The cell surface receptor which initiates this process, the asialoglycoprotein receptor, has been purified and thoroughly characterized. However, despite the extensive characterization of this receptor, its precise role in normal physiology remains controversial (2). A less well understood removal pathway is that whereby fragments of alimentary lipoproteins, called chylomicron remnants, are removed specifically, rapidly and with a very high affinity by the liver (3). It has been shown that this pathway is saturable and may involve apoprotein E as a recognition signal (4,5). This system is distinct from the well characterized low density lipoprotein (LDL) pathway which originally was described on fibroblasts (6) and is present in liver cells (7-9) in variable amounts depending on the metabolic state of the animal (10). In contrast, the remnant removal system does not undergo substantial metabolic regulation (3,11,12). Moreover, remnant removal is normal in animals and people who lack LDL receptors (13).

Many characteristics of the remnant removal system are similar to those of the asialoglycoprotein receptor, and we recently have found that both receptors and the clearance of their ligands are diminished in the ethionine treated rat (Barnard, G., Daniels, E., and Cooper, A., manuscript in preparation). Moreover, it has been shown that the lactosylation of low density lipoproteins accelerates their removal from blood and that this is due to their being transported by the hepatic asialoglycoprotein system (14). Thus, one reasonable hypothesis was that the remnant and asialoglycoprotein removal systems were related. Furthermore, because both are very abundant and active on the liver cell surface, even if the receptors are not identical, it was of interest to determine whether saturation of one of the pathways would deplete a common intermediate and thus affect the other pathway. It was to test these two questions that the present studies were undertaken.

METHODS

Animals

All rats were males of the Sprague-Dawley strain (Simonson Laboratories, Gilroy, California). They were housed in windowless rooms illuminated from 0700 to 1900 and were fed a standard chow diet. Rats used for perfusion weighed between 160-190 g, and lymph donors between 300-400 g. Retired breeders were used to prepare chylomicron remnants.

Animal Procedures and Lipoprotein Preparation

Lymph was collected from a mesenteric cannula, and chylomicrons were isolated by ultracentrifugation; chylomicron remnants were pre-

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pared exactly as previously described (3). They were iodinated by a modification of the method of McFarlane (15) as previously described (11). Their composition and the distribution of radioactivity has been reported previously (16).

Preparation of Asialoglycoproteins

Both asialooroscomucoid and asialofetuin were used in the studies. Oroscomucoid was prepared from human plasma by a standard method (17) and was found to be homogeneous by polyacrylamide gel electrophoresis by the method of Laemmli (18). Fetuin was purchased from GIBCO (Grand Island, New York). The terminal sialic acid was removed by incubating the protein in 0.1 M H_2SO_4 for 30 min at 80 C. Then the mixture was dialyzed extensively against PBS.

Liver Perfusion

Liver perfusion was performed by a modification of the method of Mortimore (19) using a sialistic coil oxygenator (20) and media containing washed human red blood cells as previously described (21). Recycling perfusion was initiated with ligand-free perfusate. Once it was clear that the liver was viable, usually after 5-10 min, this was switched to a fresh recycling perfusate containing the ligand to be tested. The outflow was switched 20 seconds after the inflow. With this protocol, there was no dilution of the ligand containing perfusate with the non-ligand containing perfusate.

Liver Plasma Membranes

Liver plasma membranes were prepared by the method of Neville (22) as modified by Pohl (23) and adapted by this laboratory (24). The characteristics of this membrane preparation have been published previously (5,24).

Binding Assays

Chylomicron remnant binding was carried out exactly as described previously (5,24,25). Asialoglycoprotein binding was assayed by the same method as chylomicron remnant binding.

RESULTS

The initial series of experiments used plasma membranes to probe whether the nature of the receptor site for the two ligands, asialofetuin and chylomicron remnants, were similar. First, consistent with work published by others (26), it was shown that ^{125}I-labeled asialofetuin bound specifically and in a saturable manner to rat liver plasma membranes (not shown). Similar saturable binding of rat chylomicron remnants of rat liver plasma membranes has been reported previously from this laboratory (5,25). Next, the ability of increasing amounts of unlabeled asialofetuin to compete for the binding of a trace of labeled asialoglycoprotein was documented (Fig. 1a). In contrast, a large excess, on a protein basis, of unlabeled chylomicron remnants failed to alter the amount of binding of the labeled trace of asialofetuin (Fig. 1a).

The converse experiment using a labeled trace of chylomicron remnants also was performed. The binding of a trace amount of labeled chylomicron remnants could be effectively diminished by an excess of unlabeled chylomicron remnants (Fig. 1b), but unlabeled

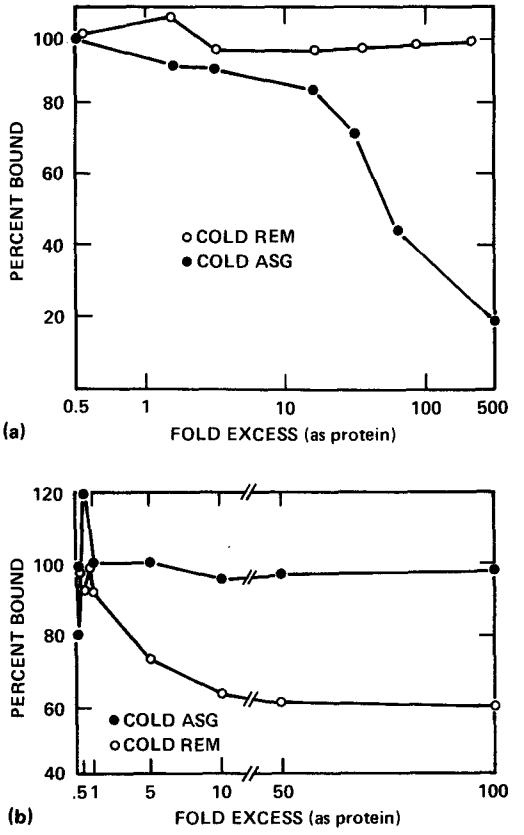

FIG. 1. Competitive binding of asialoglycoprotein and chylomicron remnants. Plasma membranes were prepared as described in Methods, and a binding assay carried out as described in Methods. The labeled trace was (a) ^{125}I-asialofetuin, 35 ng/incubation, and (b) ^{125}I-chylomicron remnants, 1 μg protein/incubation. The quantity of unlabeled competitor is indicated on the ordinate. Each point is mean of duplicate determinations in a single experiment with one batch of remnants. Duplicates agreed within 10% of each other.

asialoglycoprotein did not decrease remnant binding (Fig. 1b). The degree of nonspecific binding of remnants varied with the batch of remnants, but the assay has proven reproducible in repeated use (5,24,25). Based on this result, it is concluded that chylomicron remnants do not interact with the cell surface receptor for asialoglycoproteins nor does the putative cell surface receptor for chylomicron remnants bind asialoglycoprotein.

Following binding, ligands are internalized through coated pits by endocytosis. The liver has a considerable capacity for the removal of both asialoglycoproteins and chylomicron remnants. Thus, although the receptors are distinct, there are enough similarities in post-binding events that it is possible that the two ligands share some post-binding step which might become rate-limiting. To explore this possibility, the removal of the ligands by the intact isolated perfused liver was studied. Labeled asialofetuin or asialooroscomucoid (not shown) were removed rapidly from the isolated rat liver (Fig. 2a). However, the rate of removal was not affected by the presence of a large excess of rat chylomicron remnants (Fig. 2a). In contrast, the rate of trace removal was slowed substantially by the presence of a large excess of unlabeled asialoglycoprotein (Fig. 2a). A similar result was obtained in the converse experiment. That is, a large excess of unlabeled asialoglycoprotein did not affect the removal of a trace of labeled chylomicron remnant (Fig. 2b). This strengthens the conclusion that the two did not share a common receptor and excludes the possibility that some post-receptor step might be common to the two and become saturated when one of the two systems is operating maximally.

Interestingly, dansylcadaverine, a transglutaminase inhibitor which prevents endocytosis in several cell lines (27), did not affect either remnant or asialoglycoprotein removed in the perfused liver, even at high concentrations of 100 or 400 μM (Fig. 3).

DISCUSSION

Based on the observations presented above, several conclusions can be drawn. First, the asialoglycoprotein receptor system does not have a role in chylomicron remnant metabolism. It has been shown previously that this receptor, which is abundant on the cell surface, did not have a role in LDL metabolism (28). However, when LDL was lactosylated, it was transported by the asialoglycoprotein receptor with a much higher rate and affinity than native LDL (14) and, in fact, the metabolism of lactosylated LDL closely resembled the hepatic metabolism of chylomicron remnants. This, together with the marked similarity between the chylomicron remnant and asialoglycoprotein removal systems, made plausible the hypothesis that the two were related. This is not the case. The binding data excludes the possibility of a remnant interaction with the asialoglycoprotein receptor and makes the converse highly unlikely as well.

However, the two transport systems share several characteristics including a very high affinity and capacity and lack of metabolic regulation as well as a decrease in content during malignant degeneration of liver (Barnard, G., Daniels, E. and Cooper, A.D., manuscript in preparation). Thus, it remained possible that even if the receptors were not identical, the two systems

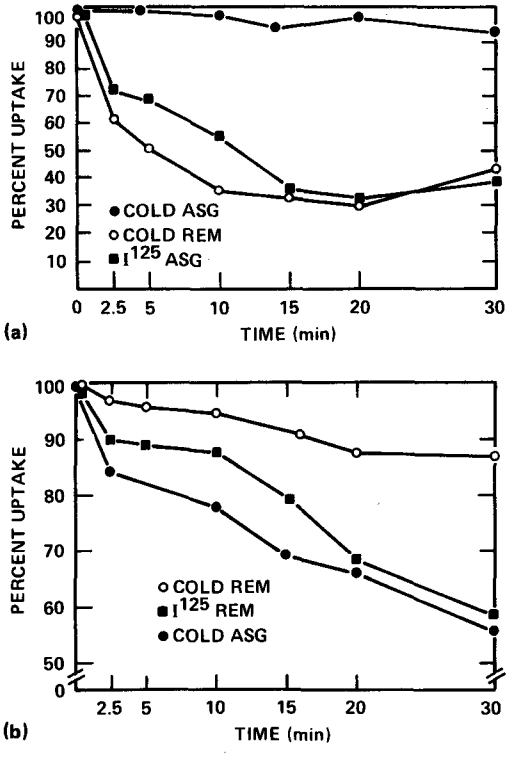

FIG. 2. Competition for removal of asialoglycoprotein and chylomicron remnants. Liver perfusion was carried out as described in Methods. After the perfusion was carried out as described in Methods. After the perfusion was established, the perfusate was changed to one which contained radiolabeled ligand and an excess of unlabeled competitor. In (a) the labeled ligand is ^{125}I-asialooroscomucoid (about 2 ng/ml); and in (b) ^{125}I-chylomicron remnants (about 1 μg/ml), the excess was 500- to 1000-fold for asialooroscomucoid and 100-fold for remnants on a protein basis.

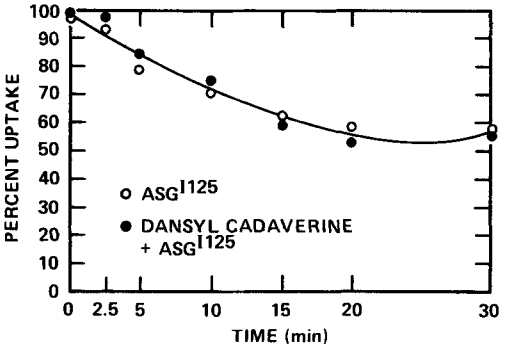

FIG. 3. Effect of dansylcadaverine on asialoglycoprotein removal. Liver perfusion was carried out as described in Methods. ^{125}I-asialooroscomucoid (about 2 ng/ml) was added to control perfusate or perfusate containing 100-400 μM dansylcadaverine. Each point is representative of duplicate determinations with a single batch of remnants. Duplicates agreed within 10% of each other.

might share some post-receptor step or steps. Under conditions where the rate of transport of one was very high, the transport of the other might be limited. Such an interaction might occur at the coated pit, during internalization or with recycling of the receptors. The perfused liver was used to study these possibilities. The results with perfused livers confirmed the conclusion regarding the distinctness of the receptors. Moreover, they demonstrated that a high rate of transport of one macromolecule does not impair the liver's ability to transport other macromolecules. It appears that the liver cell has adequate surface and sufficient metabolic energy to accommodate the simultaneous transport of these two macromolecules at high rates through similar pathways. A similar conclusion has been reached about the effect of macromolecular transport on the function of the insulin receptor (29).

The nature of the remnant removal system remains somewhat uncertain at this point. While substantial evidence has been presented that apoE is an important ligand in the system (4,5), there also is evidence for a role of the phospholipids (30) as well as other apoproteins (31,32). Thus, it is not clear whether remnant metabolism proceeds by a manner strictly analogous to that of LDL transport or whether a different or more complex system is involved.

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Long-Term Effects of High-Fat Diets on Peroxisomal β-Oxidation in Male and Female Rats

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ABSTRACT

In weanling male rats a 4-fold increase of heart triacylglycerols was observed after three days on a high-fat diet containing partially hydrogenated fish oil (PHFO). In female rats this increase was only about 50%. No significant differences were observed between female and male rats in the fatty acid composition of the accumulated lipids.

The initial level of peroxisomal β-oxidation activity was similar in male and female rats in both liver and heart. After three weeks of receiving high-fat diets, the rats showed a marked increase in peroxisomal β-oxidation activity with PHFO in the diet and less with soybean oil (SO), confirming previous studies with male rats. Catalase activity was similarly affected in hearts of both sexes.

In male rats the levels of peroxisomal β-oxidation observed after three weeks of feeding on the high-fat diets were found to be maintained, both in liver and heart, during a feeding period of three months. The response to high-fat diets in females, however, seems to be further accentuated after three months of feeding, resulting in a capacity of peroxisomal β-oxidation in liver of about three times that of the male rats when calculated on a total body-weight basis.

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INTRODUCTION

When rats are given dietary oils containing $C_{22:1}$ fatty acids, as rapeseed oil (RO) or partially hydrogenated fish oil (PHFO), a transient accumulation of lipids is observed in heart and skeletal muscles (1-3). For review, see (4).

It is believed that an enhancement of peroxisomal β-oxidation activity may, at least in part, account for the transient nature of the lipidosis (5). Thus, diets containing $C_{22:1}$ fatty acids have been shown to result in an adaptive increase of peroxisomal β-oxidation activity both in liver (6-8) and heart (9) of weanling male rats. Other high-fat diets have been found to evoke a similar, but less extensive increase in this activity (6,8,9).

Time-course experiments with diets containing PHFO have shown that in the liver of male rats the peroxisomal β-oxidation activity increases gradually up to a maximal level after three to four weeks of feeding (6,7,10). In the heart, this maximum seems to be reached somewhat faster (9). There appear to be no studies conducted on the effects on the peroxisomal β-oxidation system of prolonged feeding on high-fat diets. Furthermore, it is not clear whether these changes are confined to male rats only.

In the present study we have compared the cardiac lipidosis observed after PHFO-feeding in male and female rats. We also have studied the initial level of peroxisomal β-oxidation activity, as well as the effects of feeding different high-fat diets given for up to three mo on this system on the liver and heart of both sexes.

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EXPERIMENTAL

Materials

Dietary oils, including analytical details, were obtained from DeNoFa and Lilleborg Fabriker A/S, Fredrikstad, Norway. The fish oil was produced from capelin (*Mallotus villosus*). The fatty acid compositions are given in (8). Vitamin and salt mixtures were from ICN Pharmaceuticals, Cleveland, Ohio (vitamin diet fortification mixture, catalogue no. 904654, and U.S.P. XVII, catalogue no. 904610). Reagents used were commercial products of high purity, mostly from Sigma Chemical Co., St. Louis, Missouri. "Nyco-test-triglycerides" were obtained from Nyegaard and Co., Oslo, Norway.

Animals and Diets

Weanling rats (60 g) of the Wistar strain were purchased from Møllegaard Breeding Laboratory, Ejby, Denmark. The animals were fed a standard pellet diet for five days, then given the experimental, semisynthetic diets for up to three mos. The standard pelleted diet was delivered from Möllesentralen A/S, Oslo, Norway. The main composition of this diet is meal from soybean, barley and wheat with 4% (w/w) fishmeal and 3% (w/w) milk powder, fortified with

vitamins, amino acids and minerals. The fishmeal contained 0.3% fat and a negligible amount of 22:1 fatty acids (0.15 μg/g). The protein and fat contents were 25% and 2% (w/w), respectively. The composition of the semisynthetic diets was, in weight percentage of the total: sucrose, 20.0%; corn starch, 27.8%; casein (with 2% methionine), 20%; cellulose, 1.0%; vitamin mixture, 2.2%; salt mixture, 4.0%; dietary oil, 25%. The PHFO-diet contained 20% partially hydrogenated fish oil, and 5% soybean oil to avoid essential fatty acid deficiency (8). The rats were housed in grid-bottomed cages, two in each cage, and had free access to food and water. The climatic conditions were: 23 C, 60% relative humidity, and a 12-hr light period (7 a.m.-7 p.m.).

The 10% (w/v) liver homogenate and the peroxisome-enriched fractions from liver and heart were prepared by differential centrifugation essentially as described previously (8,9).

Enzyme Assays

Cyanide-insensitive palmitoyl-CoA-dependent NAD^+-reduction was measured spectrophotometrically as described by Lazarow (11), with the modifications introduced by Flatmark et al. (12). A Cary double beam spectrophotometer model 219 was used. The contents of the sample and the reference cuvette were identical except for palmitoyl-CoA, which was added to the sample cuvette only (final concentration 40 μM). The assay was performed at pH 8.0 at room temperature (20 C). With heart preparations, the assay was performed with freshly prepared samples, because freezing and thawing evoked a large capacity for NADH reoxidation, making spectrophotometric measurements unfeasible. Catalase (EC 1.11.1.6) activity was determined spectrophotometrically at 20 C, using the wavelength of 265.5 nm (13). The incubation medium contained 0.1% (w/w) Brij 58.

Lipid Extraction and GLC Analysis

Lipid extraction and separation of lipid classes were performed as described previously (14). Fatty acids in the triacylglycerol and phospholipid fractions were methylated by using a mixture of benzene, methanolic HCl and dimethoxypropane at room temperature overnight (15). The methyl esters were separated by gas liquid chromatography (GLC) on a nonpolar (SE 30) vitreous silica capillary column as described previously (8). Identification of major peaks was made by comparing the retention times with those of fatty acid methyl ester standards obtained from Supelco, Inc., Bellefonte, Pennsylvania.

Triacylglycerol Determination

Triacylglycerol-glycerol was determined spectrophotometrically ("Nyco-test triglycerides") according to the principles described in (16).

Protein Determination

Protein was determined by using the Folin-Ciocalteu reagent (17) with bovine serum albumin as standard.

Statistical Analysis

Student's t-test was used to evaluate the significance of differences between population means.

RESULTS

The animals in both dietary groups thrived and seemed healthy throughout the three mo experimental period, and the weight-gain curves were similar (within sexes) in the two dietary groups, as indicated in Table 1. Liver and heart weights also are included in this table. No differences were observed between the dietary treatments except for the male rats given PHFO for three mo which exhibited a statistically sig-

TABLE 1

Liver-, Heart- and Body-Weights of Rats Fed SO- or PHFO- Diets[a]

Dietary treatment	Feeding period	Female rats			Male rats		
		Liver (g)	Heart (g)	Body (g)	Liver (g)	Heart (g)	Body (g)
Pellets (4)	–	4.3 ± 0.3	0.38 ± 0.02	94 ± 5	4.7 ± 0.7	0.42 ± 0.03	102 ± 11
SO (4)	3 weeks	8.6 ± 0.1	0.72 ± 0.05	188 ± 9	12.0 ± 1.4	0.93 ± 0.05	270 ± 36
SO (6)	3 mo	9.1 ± 1.4	0.86 ± 0.06	272 ± 21	13.1 ± 1.3	1.15 ± 0.09	440 ± 45
PHFO (4)	3 weeks	8.7 ± 0.9	0.77 ± 0.02	175 ± 11	11.6 ± 1.0	0.90 ± 0.10	238 ± 18
PHFO (6)	3 mo	10.5 ± 1.2	0.88 ± 0.10	272 ± 21	17.0 ± 1.8[b]	1.17 ± 0.08	452 ± 34

[a]Rats were fed a standard pelleted diet for 5 days and then given the semisynthetic diets for the time indicated. The tabulated values represent means ± S.D. for the number of animals indicated in parentheses. Abbreviations: SO, soybean oil; PHFO, partially hydrogenated fish oil.

[b]Significantly different ($p<0.01$) from the values obtained with the animals given the SO-diet for the same period of time.

nificant increase in liver weight compared to the SO-fed animals.

Heart Lipidosis

From the results presented in Table 2 it can be seen that in young rats of the Wistar strain, PHFO-feeding led to a four-fold increase in the amount of triacylglycerols (TG) in male rats after three days. In female rats the increase was only about 50%. No significant differences were observed, however, between female and male rats in the fatty acid composition of heart lipids (Table 3). The percentages of 22:1 and 20:1 fatty acids in the accumulated TG were in both groups very similar to that of the dietary oil. For reference, the values from the animals fed

TABLE 2

Heart Triacylglycerols (TG) in Male and Female Rats[a]

Dietary treatment	Feeding period	Female rats TG (nmol·g heart^{-1})	Male rats TG (nmol·g heart^{-1})
Pellets (4)	–	8.3 ± 2.3	12.8 ± 0.6
PHFO (4)	3 days	13.6 ± 4.6	47.5 ± 14.7[b]

[a]Rats were fed a standard pelleted diet for five days and then given the semisynthetic PHFO-diet. The tabulated values represent means ± S.D. for the number of animals indicated in parenthesis. Abbreviation: PHFO, partially hydrogenated fish oil.

[b]Significantly different ($p<0.01$) from the values obtained in the animals given the standard pelleted diet.

TABLE 3

Fatty Acid Composition (%) of Heart Lipids in Rats Fed a PHFO-Diet for 3 Days[a]

Fatty acid	Triacylglycerols (TG)		Phospholipids (PL)	
	Female	Male	Female	Male
14:0	2.9 ± 0.6	2.9 ± 0.6		
16:0	17.4 ± 2.0	17.4 ± 2.6	11.2 ± 0.7	11.7 ± 0.6
16:1	3.9 ± 0.7	4.2 ± 1.2	0.8 ± 0.1	1.0 ± 0.2
18:0	4.8 ± 0.4	4.6 ± 0.4	19.0 ± 0.8	17.7 ± 0.8
18:1 isom.	8.0 ± 0.5	6.8 ± 0.3	7.5 ± 0.2	6.4 ± 0.2
	15.3 ± 1.2	16.0 ± 1.6	3.8 ± 0.2	4.1 ± 0.4
18:2	12.0 ± 1.1	11.4 ± 0.7	19.6 ± 1.1	20.9 ± 1.5
20:0	1.2 ± 0.2	1.1 ± 0.2	0.3 ± 0.1	0.3 ± 0.1
20:1 isom.	4.7 ± 0.8	4.7 ± 0.8	0.7 ± 0.1	0.5 ± 0.2
	4.5 ± 0.8	6.2 ± 1.0	0.8 ± 0.1	0.8 ± 0.1
20:4	0.8 ± 0.2	0.8 ± 0.2	16.8 ± 0.5	16.2 ± 1.2
22:0	1.1 ± 0.3	0.9 ± 0.5	0.2 ± 0.1	
22:1 isom.	6.1 ± 0.8	4.3 ± 1.3	0.7 ± 0.1	0.8 ± 0.3
	5.3 ± 0.9	3.6 ± 0.7	0.4 ± 0.2	0.5 ± 0.1
22:5	0.6 ± 0.2	0.5 ± 0.2	1.3 ± 0.1	1.3 ± 0.3
22:6	1.6 ± 0.5	1.4 ± 0.4	8.5 ± 0.7	8.3 ± 1.7

[a]Rats were fed on a standard pelleted diet for five days, and then given the semisynthetic PHFO-diet for three days. Heart lipis were extracted and analyzed by GLC as described in the Experimental section. Abbreviation: PHFO, partially hydrogenated fish oil.

TABLE 4

Fatty Acid Composition (%) of Heart Lipids in Rats Fed a Standard Pelleted Diet for 5 Days

Fatty acid	Triacylglycerols (TG)		Phospholipids (PL)	
	Female	Male	Female	Male
14:0	–	–	–	–
16:0	2.9 ± 1.3	2.7 ± 1.0	14.2 ± 0.5	15.4 ± 0.3
16:1	3.9 ± 0.7	4.2 ± 0.9	0.3 ± 0.1	–
18:0	7.0 ± 0.9	5.0 ± 1.0	21.7 ± 0.9	19.9 ± 0.4
18:1 isom.	3.8 ± 0.4	2.8 ± 0.5	4.1 ± 0.6	4.2 ± 0.3
	23.9 ± 4.2	24.9 ± 1.3	3.6 ± 0.5	4.4 ± 0.3
18:2	22.5 ± 1.9	23.7 ± 3.9	23.1 ± 5.0	24.1 ± 1.5
20:0	–	–	–	–
20:1 isom.	–	–	–	–
20:4	–	–	15.9 ± 2.1	13.5 ± 0.4
22:0	–	–	–	–
22:1 isom.	–	–	–	–
22:5	0.50 ± 0.1	0.49 ± 0.1	1.3 ± 0.1	1.6 ± 0.2
22:6	1.52 ± 0.1	1.47 ± 0.1	9.5 ± 2.3	9.3 ± 0.7

the standard pelleted diet for five days are shown in Table 4.

Peroxisomal β-Oxidation Activity in Liver

Peroxisomal β-oxidation activities observed in liver are presented in Figure 1a, as calculated per mg of protein in the peroxisome-enriched fractions (specific activity) and per whole liver (total activity). High-fat feeding for three weeks led to an enhancement of the peroxisomal β-oxidation activity in male rats; this was most pronounced with the 22:1 fatty acid-containing diet (PHFO), confirming earlier findings (6-8). Moreover, the results from the present study

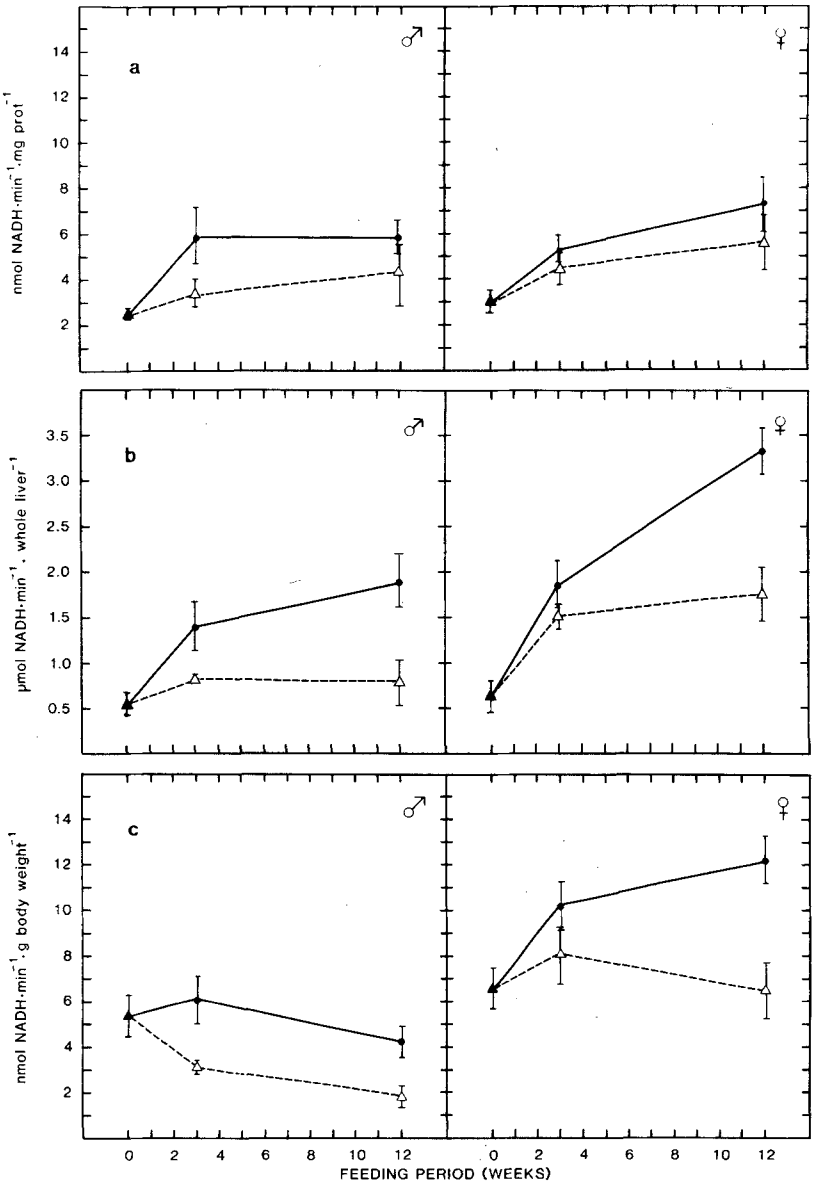

FIG. 1. Effect of dietary treatment and feeding period on peroxisomal β-oxidation activity in the liver. Rats were fed a standard pelleted diet for five days and then given the semi-synthetic SO and PHFO diets for the time indicated. Subcellular fractions were prepared, and peroxisomal β-oxidation activity determined as described in the Experimental section. Abbreviations: SO, soybean oil (△); PHFO, partially hydrogenated fish oil (●). The given values represent means ± S.D. Number of animals as in Table 1.

show that this enhanced level of peroxisomal β-oxidation is maintained upon prolonged feeding (three mo) on these diets.

Female rats also revealed an increase in peroxisomal β-oxidation after three weeks of feeding, but in these animals the difference between the values observed in the PHFO and SO groups was not statistically significant. In the females the response to PHFO seemed to be further accentuated after three mo of feeding.

Young, newly weaned male and female rats given a standard pelleted diet revealed similar activities of peroxisomal β-oxidation in liver. Furthermore, no marked differences in specific activities were observed between sexes during the course of the feeding experiment.

In total liver activity, however, significantly higher values were observed in female rats fed for three weeks and three mo on the SO-diet, and for three mo on the PHFO-diet (Fig. 1b). When calculated on a body weight basis, this resulted in about a three-fold higher activity of peroxisomal β-oxidation in female rats than in male rats after three mo of feeding. These differences were not as pronounced after three weeks of feeding, but even at that time, female rats revealed significantly higher values in both dietary groups when related to body weight (Fig. 1c).

Catalase activity was only slightly affected in liver (results not shown), confirming earlier findings (7).

Peroxisomal β-Oxidation and Catalase Activity in Heart

We have reported previously that high-fat feeding also led to increased (micro)peroxisomal β-oxidation in the heart of male rats (9). This is confirmed by the results presented in Figure 2a. As in the liver, the effects on the specific activity were similar in male and female rats during the first weeks of feeding. In the heart, however,

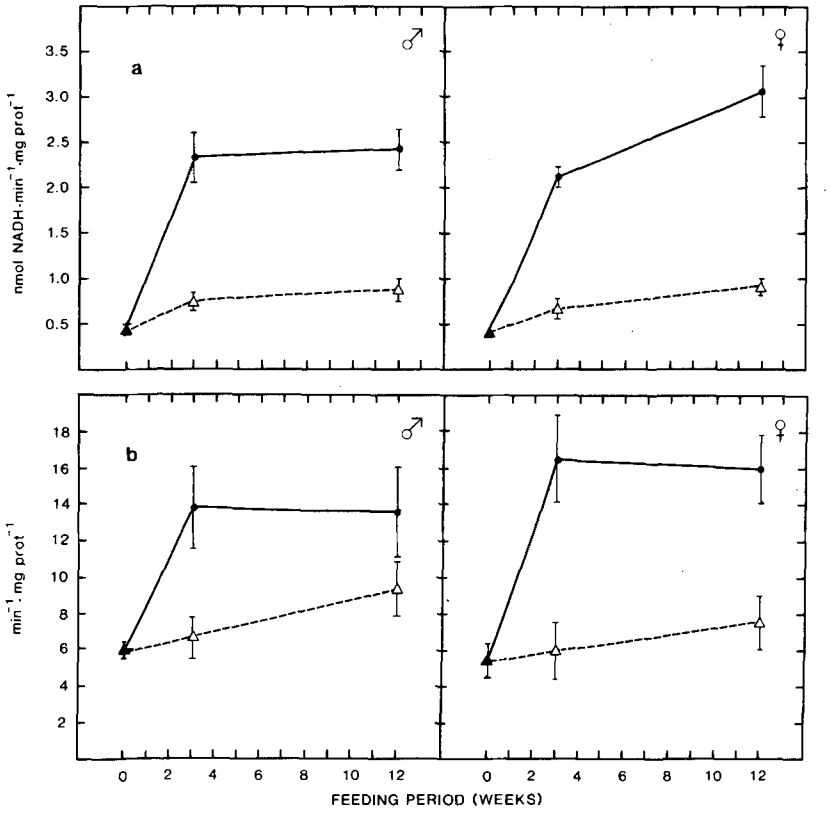

FIG. 2. Effect of dietary treatment and feeding period on peroxisomal β-oxidation (a) and catalase activity (b) in the heart. Rats were fed a standard pelleted diet for five days and then given the semisynthetic SO and PHFO diets for the time indicated. Subcellular fractions were prepared and peroxisomal β-oxidation and catalase activity determined as described in the Experimental section. Abbreviations: SO, soybean oil (△); PHFO, partially hydrogenated fish oil (●). The given values represent means ± S.D. Number of animals as in Table 1.

a statistically significant increase was observed in female rats given the PHFO diet between three weeks and three mos.

In a previous study we also observed that in rat heart the catalase activity was strongly enhanced by PHFO-feeding, in contrast to what was found in the liver (9). This was confirmed by the results presented in Figure 2b, where a 2.5- and three-fold increase was observed in males and females, respectively. The catalase activity also was enhanced by the SO-diet, although to a lesser extent. In these animals, however, a gradual increase was observed up to three mo of feeding.

DISCUSSION

In this study a markedly lower lipid accumulation was observed in female than in male rats when given PHFO in the diet. In studies using high-erucic rapeseed oil diets, no such difference between males and females has been observed (1-3). The reason for this discrepancy was not readily evident, but because these studies were performed with Sprague-Dawley rats it might be related to strain as well as to dietary differences.

A large body of evidence is accumulating that an enhancement of the peroxisomal β-oxidation system may be of significance in normalization of the lipid levels in the heart. In light of the great difference observed in lipid accumulation in males and females, the gross similarity in the response of the peroxisomal β-oxidation system during the first weeks of PHFO-feeding may seem somewhat contradictory. However, as shown in Figure 1c, when calculated on a body weight basis, female rats revealed an activity about twice as high as the males after three weeks on the PHFO diet. This would imply a higher capacity of peroxisomal β-oxidation in relation to the amount of dietary oil ingested.

Most studies on peroxisomes and peroxisomal β-oxidation have been conducted using male rats. It has been reported that the hypolipidemic drugs bezafibrate and clofibrate, both well known inducers of peroxisomal β-oxidation activity in male rats, result in enhancement of this activity in female rats also. However, higher doses seem to be needed in female rats (18,19). Krahling observed no differences in peroxisomal β-oxidation between conventionally fed male and female rats (20).

It is evident from the results of the present study that high-fat diets also increase peroxisomal β-oxidation activity in female rats, and that the responses on the whole are similar to those observed in males. When the resulting peroxisomal β-oxidation activities are related to organ or body weight, however, significantly higher values are found in females. This effect was most pronounced after three mo of feeding. To the best of our knowledge, no comparable data on peroxisomal β-oxidation exist, but significantly higher activities of carnitine-acetyl transferase have been observed in female rats in long-term studies with clofibrate or bezafibrate (18,19). The explanation for these differences was not readily evident, but sex hormones have been reported to influence catalase activity (21).

In conclusion, the increase in peroxisomal β-oxidation activity due to high-fat feeding seems to be an adaptation encountered in the organs of both sexes. Further, it seems to persist as long as the "inducing" dietary regimen is continued. The large difference between male and female rats in heart TG-accumulation after three days may be related to a somewhat higher capacity of peroxisomal β-oxidation in females on the basis of body weight. Other metabolic conditions may, however, be of importance in determining the level of TG accumulated when rats are given high-fat diets rich in 22:1 fatty acids.

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Fatty Acids and Fatty Alcohols of Wax Esters in the Orange Roughy: Specific Textures of Minor Polyunsaturated and Branched-Chain Components

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ABSTRACT

Open-tubular gas chromatography was carried out on fatty acids and alcohols obtained from wax esters of the orange roughy, *Hoplostethus atlanticus*, caught at sea off New Zealand. The major (above 5%) components were 16:1(n-7), 18:1(n-9) and (n-7), 20:1(n-9) and (n-7), and 22:1(n-11, n-13) as fatty acids, and 16:0, 18:0, 18:1(n-9), 20:1(n-9) and (n-7), and 22:1(n-11, n-13) as fatty alcohols. The total percentages of the minor components were 10% in the acids and 26% in the alcohols. The 22:1/20:1 ratio of the fatty alcohols obtained in this study was less than 1.0, although the ratio for the Atlantic orange roughy has been reported as being greater than 1.0. The contents of polyenes were as low as 2.48% in the acids and 0.95% in the alcohols, but their compositions showed some specific features. The percentages of the C_{16}-C_{22} dienes in the total polyenes were remarkably high, 57.7% of these acids and 53.1% of these alcohols. The most important dienes were 18:2(n-6) in the acids and 20:2(n-6) in the alcohols.
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INTRODUCTION

The fatty oil of the deep-sea teleost, the orange roughy, contains wax esters as the major components. It could be used as a substitute for sperm whale oil and jojoba oil (1). Mori et al. reported the fatty acid and alcohol compositions of wax esters from the flesh of *Hoplostethus gilchristi* caught at 1000 m depth off New Zealand in 1978 (2). Subsequently, Hayashi and Takagi reported the acid and alcohol compositions of wax esters from the flesh of *H. atlanticus* caught in the same waters (3). Since then, four papers (4-7) have been published on the compositions of orange roughy wax esters. In these studies, the major components, saturated and monounsaturated straight-chain acids and alcohols, were the main subjects of investigation.

The present paper also describes the detailed compositions of the minor polyunsaturated and branched-chain fatty acids and alcohols from wax esters of orange roughy caught off New Zealand. In addition, the compositions of the positional isomers of the monounsaturated components are reported for comparison with those shown in previous papers (6,7).

MATERIALS AND METHODS

Materials and Separation of Wax Esters

The sample oil was produced, with meal, from orange roughy caught in deep water off New Zealand by a trawler in 1983. The crude oil obtained from Nikko Chemicals Co., Tokyo, in February, 1984 was stored at about -20 C for 3 mo until used in this study.

Separation of wax esters from the oil was accomplished by thin layer chromatography (TLC) with Silica Gel G coated at 0.5 mm in thickness, using benzene/n-hexane (3:2, v/v) as a developing solvent. Rhodamine 6G solution in ethanol was used as a visualizing reagent.

Preparation of Methyl Esters and Acetates

The wax esters were converted to fatty acid methyl esters and fatty alcohols by direct transesterification with 5% HCl in methanol/benzene (1:2, v/v) heated at 80 C in screw-cap test tubes for 3 hr under nitrogen. The methyl esters and alcohols were separated by TLC with Silica Gel G plates with development in n-hexane/ether (85:15, v/v). The alcohol fraction was converted into acetates by heating with acetic anhydride/pyridine (1:1, v/v) at 80 C in screw-cap centrifuge tubes for 1 hr under nitrogen.

Gas Liquid Chromatography (GLC)

Fatty acid and alcohol compositions were obtained by GLC of the methyl esters and acetates using a Shimadzu GC 6 AMPF instrument (Shimadzu Seisakusho Co., Kyoto) equipped with a dual FID detector and a glass capillary WCOT column (50 m × 0.28 mm id) coated with SP 2300 (Supelco Inc., Bellefonte, Pennsylvania). The carrier gas was H_2 with a flow rate of 0.5 ml/min. The column temperature was 200 C, with the detector and sample inlet

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TABLE 1

Composition of Fatty Acids and Alcohols of Orange Roughy Wax Esters

This study			Previous study (3)[a]		
Component	Acid	Alcohol	Component	Acid	Alcohol
13:0	0.04	0.03	13:0	–	–
14:0	0.93	2.08	14:0	1.4	2.1
15:0	0.05	0.54	15:0	0.1	0.7
16:0	0.81	22.16	16:0	2.1	28.3
17:0	0.50	0.49	17:0	0.4	0.2
18:0	0.28	5.95	18:0	0.7	7.6
19:0	–	0.17	19:0	–	0.2
20:0	0.03	0.52	20:0	–	0.5
21:0	–	0.06	21:0	–	–
22:0	–	0.12	22:0	–	0.2
15:0 iso	–	0.16	15:0 iso	–	0.2
anteiso	0.03	0.03	anteiso	0.1	–
16:0 iso	0.01	0.24	16:0 iso	–	0.4
anteiso	–	0.02	anteiso	–	–
17:0 iso	–	0.58	17:0 iso	0.3	0.3
anteiso	–	0.32	anteiso	–	0.8
18:0 iso	–	1.05	18:0 iso	–	0.5
19:0 iso	–	0.04	19:0 iso	–	–
20:0 iso	–	0.23	20:0 iso	–	–
anteiso	–	–	anteiso	0.2	0.2
Total (sat.)	2.68	34.78		5.5	42.0
14:1 (n-5)	0.33	–	14:1	0.3	–
16:1 (n-9)	–	0.09	16:1	13.4	1.9
(n-7)	11.54	0.78			
17:1 (n-8)	0.73	0.30	17:1	0.5	0.5
18:1 (n-9)	51.11	11.35	18:1	59.4	16.2
(n-7)	4.91	2.76			
(n-5)	0.12	0.47			
19:1 (n-8)	0.29	0.33	19:1	0.6	0.5
20:1 (n-9)	11.56	16.17	20:1	12.4	17.1
(n-7)	5.54	9.10			
(n-5)	0.68	1.23			
22:1 (n-11)	5.87	12.98	22:1	5.4	14.5
(n-9)	1.43	3.80			
(n-7)	0.07	1.31			
23:1 (n-8)[b]	–	0.11	–	–	–
24:1 (n-11)	–	0.51	–	–	–
(n-9)	0.67	2.97	–	–	–
Total (monoene)	94.84	64.26		92.6	53.9
16:2 (n-6)	0.08	–	17:2	–	0.1
18:2 (n-6)	1.00	0.12	18:2	1.3	0.8
(n-3)	0.05	–	–	–	–
18:3 (n-6)	0.04	–	18:3	0.4	–
(n-3)	0.20	0.09	19:2	–	0.2
18:4 (n-3)	0.18	–	–	–	–
20:2 (n-6)	0.14	0.28	20:2	0.1	1.8
20:3 (n-6)	0.05	–	–	–	–
(n-3)	0.13	–	–	–	–
20:4 (n-6)	0.08	0.18	20:4	0.1	–
(n-3)	0.19	0.03	–	–	–
20:5 (n-3)	0.21	–	–	–	–
22:2 (n-6)	0.04	0.11	22:2	–	1.1
22:5 (n-3)	0.03	0.02	–	–	–
22:6 (n-3)	0.06	0.13	24:2	–	0.1
Total (polyene)	2.48	0.96		1.9	4.1

[a]Wax esters were separated from the total lipids extracted from the muscle of a male *H. atlanticus* caught from ca. 940 m depth off New Zealand. The extraction was done by method of Bligh and Dyer. GLC was done on 1.5 m × 3 mm columns packed with 10% DEGS on Chromosorb W and with 5% SILAR 10 C on Gas Chrom Q.

[b]Tentatively identified by plotting of carbon numbers vs. log relative retention times for the n-8 series of monoenoic acid methyl esters.

at 230 C. Peak area percentages were obtained with a Shimadzu integrator C-R2AX.

The component of each peak was identified as shown in Table I on the basis of agreement of the retention data with those of reference, methyl esters of sea urchin fatty acids (8,9), and acetates of fatty alcohols obtained by $LiAlH_4$ reduction of the same methyl esters. The log plot procedure and the systematic separation factor procedure (10) were used concurrently for the identification.

Argentation-TLC ($AgNO_3$-TLC)

The fatty acid methyl esters and alcohol acetates also were fractionated according to their degree of unsaturation on silver nitrate-impregnated layers of Silica Gel G by developing with ethyl acetate/n-hexane (1:9, v/v).

Hydrogenation of Methyl Esters and Acetates

The methyl esters and acetates were completely hydrogenated by the usual procedures described in a previous paper (3), using 5% palladium on carbon as a catalyst.

$AgNO_3$-TLC Analysis with the Chromarod-Iatroscan System

The procedures described in previous papers (11-13) were used for the analysis. Chromarods-SII cleaned by being passed through a flame were immersed in a 2.5% solution of silver nitrate in acetonitrile for 15 min, and then activated by heating in an oven at 120 C for 3 hr. The rods were spotted with 1 μl of 1-10% sample solution in chloroform, and developed with ethyl acetate/hexane (0.5:9.5, v/v) for the separation of saturates, monoenes, dienes and other polyenes. After developing, the rods were air-dried and then scanned with a flame ionization detector in an Iatroscan TH-10 instrument (Iatroscan Laboratories, Tokyo). Peak areas were measured with a Chromatopac C-R1A (Shimadzu Seisakusho).

RESULTS AND DISCUSSION

Major Fatty Acid and Alcohol Components

Fatty acids and alcohols found as components of wax esters in the orange roughy oil by WCOT GLC are shown in Table 1. There were six major (above 5%) components in each case. They were 16:1(n-7), 18:1(n-9) and (n-7), 20:1 (n-9) and (n-7), 22:1(n-11, n-13) as fatty acids, and 16:0, 18:0, 18:1(n-9), 20:1(n-9) and (n-7), and 22:1(n-11, n-13) as fatty alcohols. The total contents of the major components, 90% for the acids and 74% for the alcohols, show that the proportion of minor components was higher in the alcohols.

Saturated Acids and Alcohols

A low proportion of saturated acids is a characteristic of the wax esters from orange roughy oils. The content of saturated acids in the total acids has not exceeded 5% in any of the previous papers (1-7), and the figure was less than 1% in this study. The content of saturated alcohols obtained in this study was markedly lower than that reported in our previous paper (3), as shown in Table 1, while the monounsaturated alcohol content in this study was remarkably higher. The acid and alcohol compositions in this study were somewhat analogous to those reported by Mori et al. (2). The 16:0/18:0 ratios reported for the alcohols of orange roughy wax esters in the literature are in the range of 3 to 5, except for a figure of less than 1.0 in the data reported by Buisson et al. (1). The ratio was about 4 in this study.

The content of branched components was greater in the alcohols than in the acids. The total content of branched saturated alcohols was 2.6% of the total alcohols. We verified this by total hydrogenation and reanalysis. The detailed composition of hydrogenated acetates of the alcohols is shown in Table 2. The comparison of the unhydrogenated component (from Table 1) and of the total corresponding components after hydrogenation, within which there is a 2.4% content of branched alcohols, con-

TABLE 2

Composition of Hydrogenated Products of Fatty Acids and Fatty Alcohols

Component	Found		Calculated[a]	
	Acid	Alcohol	Acid	Alcohol
13:0	0.07	0.50	0.04	0.03
14:0	0.97	1.83	1.26	2.08
15:0	–	0.53	0.05	0.54
16:0	11.67	22.00	12.34	23.03
17:0	0.85	0.86	1.23	0.79
18:0	56.30	20.54	57.80	20.61
19:0	0.68	0.92	0.29	0.50
20:0	19.25	27.87	18.85	27.65
21:0	0.30	0.43	–	0.09
22:0	8.02	18.57	7.44	18.48
23:0	–	–	–	0.11
24:0	1.17	3.57	0.67	3.43
iso-15:0	–	0.17	–	0.16
ai:15:0	–	0.13	0.03	0.03
iso-16:0	–	0.25	0.01	0.24
ai-16:0	–	–	–	0.02
iso-17:0	0.72	0.48	–	0.58
ai-17:0	–	0.28	–	0.32
iso-18:0	–	0.78	–	1.05
iso-19:0	–	0.14	–	0.04
iso-20:0	–	0.15	–	0.23

[a]Calculated from the data in Table 1.

firms that the branched alcohols in the wax esters are mostly saturated. Except for one report (3), an appreciable content of branched alcohols has not been reported in any previous paper on orange roughy oils (1,2,4-7). However, capillary GLC has shown more branched chain alcohols than acids in wax esters of the white barracudina *Paralepsis rissoi Krøyeri* Bonaparte 1840 from Nova Scotian waters (14).

Monounsaturated Acids and Alcohols

The monoenes, 16:1(n-7), 18:1(n-9) and (n-7), 20:1(n-11), 22:1(n-11), were reported as being the major components of the acids and alcohols in the analysis of orange roughy wax esters by Sargent et al. (7). Additional minor monoenes, 18:1(n-5), 20:1(n-7) and (n-5), 22:1 (n-9) and (n-7), and 24:1(n-9), were detected by WCOT GLC in this study. Occurrence of 24:1(n-11) and (n-13) was reported by Body et al. (6) from the results of gas liquid chromatography-mass spectroscopy of di(trimethylsilyl) ethers of diols which were obtained by oxidation, but they were not detected in this study. Under the conditions of the WCOT GLC used in this study, the 22:1(n-13) component was included with the 22:1(n-11) recorded in Table 1.

The 22:1/20:1 ratios in the fatty alcohols were found to be greater than 1.0 in Atlantic orange roughy wax esters by Sargent et al. (7). In contrast, the 22:1/20:1 ratios are 1.0 or less in Pacific orange roughy wax esters (1-6). The ratio obtained in this study also was obviously less than 1.0. This variation of 22:1/20:1 alcohol ratios might depend on the biosynthetic mechanisms of chain elongation and desaturation of shorter acids and alcohols, de novo synthesis of 20:1 and 22:1 acids and their conversion to alcohols, and the catabolism of the acids and alcohols as energy sources, as described in the literature (7,15). The compositions of monounsaturated fatty alcohols in certain fish body lipids and commercial fish oils were compared with those in copepods, and 22:1 alcohols in the wax esters of copepods are assumed to be precursors of the 22:1 fatty acids of fish depot fats, specifically of the dominant 22:1(n-11) isomer (16). In this study, the major 22:1 acid in the wax esters was 22:1(n-11) acid, and it may be formed mainly from 22:1(n-11) alcohol. Therefore, the high proportions of 22:1/20:1 of the alcohols compared to those of the acids reported in the literature (1-6), and obtained in this study, are assumed to be due to the low conversion of the 22:1 alcohols to the 22:1 acids in the orange roughy.

High n-6/n-3 Ratios of Polyunsaturated Acids and Alcohols

Compositions of polyunsaturated acids and alcohols in orange roughy wax esters have been reported in only a few papers (2,3,7), probably due to their low content. In this study, the detailed composition of the polyunsaturated components was obtained by WCOT GLC as shown in Table 1. The results showed some specific features which were different from the polyunsaturated components of ordinary marine fish oils (triacylglycerols) where n-3 fatty acids are dominant. The proportions of n-6 components apparently were higher in the polyunsaturated acids and alcohols of the orange roughy used in this study. The alcohols favor 18:2(n-6), 20:2 (n-6), 22:2(n-6), and 20:4(n-6) relative to the C_{20} and C_{22} n-3 series, except for the relatively important 22:6(n-3). On the contrary, the acids favor the C_{18} and C_{20} n-3 series relative to the C_{18} and C_{20} n-6 series, except for 18:2(n-6), as shown in Table 1.

Abundance of Dienes in Polyunsaturated Acids and Alcohols

The second specific feature of the polyunsaturated acids and alcohols of orange roughy wax esters is their high proportion of diunsaturated components, as shown in Table 1. Dienoic acids, such as 16:2(n-6), 18:2(n-6) and (n-3), and 20:2(n-6) and (n-3), have been found as minor components of marine fish oils, but they do not seem to accumulate in fish to the extent of more than 1 or 2% of the total fatty acids (17,18), and the dienoic acid content is below 10% in the polyenoic acids. The percentages of the dienes in the polyunsaturated acids and alcohols of the orange roughy wax esters were at usually high levels, 57.7 and 53.1%, respectively, in this study. Linoleic acid was the most important of the dienoic acids, while 20:2(n-6) alcohol was the most important diunsaturated alcohol. Linoleic acid [18:2(n-6)] was the only dienoic acid found in wax esters of the orange roughy caught off the west coast of Britain (7), and its percentage in the total of polyenoic acids was 33%. The diene fraction of the fatty acid methyl esters and alcohol acetates separated by $AgNO_3$-TLC revealed peaks listed as the dienes in Table 1. Percentages of the unsaturates in acids and alcohols obtained by $AgNO_3$-TLC by Chromarod-Iatroscan are shown in Table 3. This independent method supports the abundance of diunsaturated components in the polyunsaturated acids and alcohols of orange roughy wax esters.

TABLE 3

$AgNO_3$-TLC Analysis of Polyenoic Acids and Alcohols Based on the Unsaturation with an Iatroscan

	Fatty acid %		Fatty alcohol %	
	Found	Calcd.[a]	Found	Calcd.[a]
Dienes	50.9	52.8	46.8	53.1
Trienes	–[b]	–	8.1	9.4
Other polyenes	49.1[c]	47.2	45.1	37.5

[a]Calculated from the data in Table 1.

[b]The trienoate peak for acids could not be separated from the more highly unsaturated polyenoate peak.

[c]Including percentages of the trienoates.

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The Monounsaturated Acyl- and Alkyl- Moieties of Wax Esters and Their Distribution in Commercial Orange Roughy *(Hoplostethus atlanticus)* Oil

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ABSTRACT

Wax esters were isolated from commercial orange roughy (*Hoplostethus atlanticus*) oil by column chromatography and fractionated by argentation thin layer chromatography. Following transesterification, the resultant fatty acid methyl esters and fatty alcohols were analyzed by gas chromatography. Both acyl- and alkyl-moieties were mainly of the monoene structure within the 16:1-22:1 range. After derivatization, the positions of the double bonds of even numbered fatty acid and fatty alcohol isomers were located by chromatography-mass spectrometry and compared.

Results of these positional analyses indicate that the primary desaturation reactions takes place in the Δ9 position of pre-existing (C_{14} to C_{24}) acyl chains. It is proposed that acyl components from 18:1 are subjected to chain elongation to form a mixture of 24:1 isomers as the final product. Apart from the 24:1 acyl moiety of the wax esters, in which the double bond was almost exclusively in the Δ15 position, de novo biosynthetic reactions on acids and alcohols appear to yield related acyl- and alkyl-moieties of resynthesized wax esters.
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INTRODUCTION

The oil from the flesh of the deep-sea fish, orange roughy (*Hoplostethus atlanticus*) is unusual in that it is very rich (approx 95%) in wax esters (1-3). Most other fish oils contain mainly triacylglycerols (4). A study of the chemical and physical properties of orange roughy oil (2) showed that it readily could replace sperm oil and substitute for jojoba seed oil in many forms (cosmetic, sulfurized derivative for lubrication, polish waxes and textiles) of industrial usage. Currently five orange roughy oil plants have been established in New Zealand, and they regularly can produce approximately 1000 tons per year. Nearly 90% of this oil is exported to Japan, Europe and Australia, while the remainder is used on the local market as fuel.

In this communication, further details of the structure of the wax esters are presented. The positions of the double bonds in the individual monounsaturated fatty acid and alcohol moieties with different carbon chain-lengths are reported.

MATERIALS AND METHODS

The wax ester portion of a commercial orange roughy oil was isolated by silicic acid column chromatography (LC) as described previously (5,6). These wax esters were further resolved according to their degree of unsaturation by argentation (10% [w/w] silver nitrate on silica gel) thin layer chromatography (TLC) with benzene-hexane (1:1, v/v) development (7). Each wax ester fraction was analyzed by gas chromatography (GC) with a dual flame Pye 104 instrument fitted with a packed column of 3% JXR on Gas Chrom Q (65 × 0.2 cm i.d.).

Transesterification of the wax ester fractions with sodium methoxide in methanol (8), followed by diazomethane treatment (9), gave mixtures of methyl esters and alcohols. These were separated by silicic acid LC with chloroform as solvent (6). Each methyl ester and alcohol fraction was analyzed by GC using packed columns of 10% EGSS-X on Chromosorb W (300 × 0.2 cm i.d.) and 3% JXR on Gas Chrom Q (180 × 0.4 cm i.d.) respectively. All technical details and identification procedures have been outlined earlier (6,10).

Isolation of Individual Monounsaturated Methyl Esters and Acetates

A portion of the original wax esters was transesterified with boron trifluoride in methanol (11) and the resultant methyl ester and alcohol components separated by LC as described above. The alcohol fraction was divided into two parts. One part was acetylated by pyridine-acetic anhydride (12) and the other oxidized by chromic acid (13) to acids which subsequently were methylated. The saturated and unsaturated components of the methyl esters and acetates were separated by argentation LC (10% [w/w] silver nitrate on silicic

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acid) using petroleum ether-toluene (80:20, v/v) as solvent (14). Monoene methyl esters and acetates were resolved according to their carbon chain-lengths by reverse-phase column chromatography. The column packing was prepared by bonding 10% (w/w) C_{18} hydrocarbon onto silicic acid (15). Monoenes were eluted in order of increasing chain-length with methanol-water mixtures from 80:20, v/v to 100:0, v/v. The column eluant was monitored by TLC and GC.

Derivatization of Monoenes

The unsaturated methyl ester fractions were oxidized with performic acid (16) and the hydroxy formates transesterified with acidic methanol. The resulting diols were converted to their corresponding di(trimethylsilyl)ether with di(trimethylsilyl)trifluoroacetamide. Unsaturated acetate fractions were oxidized with osmic acid (17), giving the corresponding osmate esters. Subsequent reaction with hydrogen sulphide (18) gave the diol products which were converted to their di(trimethylsilyl)ethers. The silylated fractions (esters and alcohol acetates) were examined by GC. A Hewlett-Packard Model 5840A equipped with flame ionization detectors fitted with a packed column of 10% OV-101 on Gas Chrom Q (200 × 0.2 cm i.d.) was used with an oven temperature of 220 C. A shorter column (50 × 0.2 cm i.d.) containing 3% OV-101 on the same solid support was required for the analysis of longer chain-length components.

Gas Chromatography-Mass Spectrometry (GC-MS)

The silylated methyl and acetate esters were characterized by GC-MS using a double beam Kratos MS30 mass spectrometer interfaced to a Pye 104 gas chromatograph via an all glass S.G.E. (Scientific Glass Engineering Pty Ltd., Ringwood, Victoria, Australia) single stage jet separator held at 270 C. The above OV-101 columns were used for the analysis of compounds up to C_{20}, using helium as the carrier gas. The GC included a variable S.G.E. effluent splitter which directed 30% of the column effluent to the flame ionization detector and the remainder to the MS. Pure compounds were inserted by a direct probe. Low resolution electron impact mass spectra were recorded at 70 eV and at a scan rate of 3 sec/decade. The source temperature was 220 C.

RESULTS

The distribution of wax esters in this commercial orange roughy oil, according to their degree of unsaturation (either in the acyl- or alkyl-moiety) as obtained by argentation TLC and GC, is given in Tables 1 and 2. Monoenoic wax esters can be partially resolved (Bands A and B) according to their total chain-lengths. Components in which both acyl- and alkyl-moieties are monounsaturated (Band C) represented 62% of the wax esters.

Band A, in which the principal esters contained 36 to 40 carbon atoms, migrated further on the TLC plate than did Band B. Almost three quarters of Band B was comprised of esters containing 34 and 36 carbon atoms. This implies that components of longer chain-length are less strongly bound to the silver nitrate in the silicic acid absorbent than those of shorter chain-length. However, this phenomenon probably is related more to the polarity of the esters

TABLE 1

Fractionation of Orange Roughy Wax Esters by $AgNO_3$-Impregnated Thin Layer Chromatography

Band[a]	Chain length[b] 32	34	36	38	40	42	44	46	Number of double bonds	Rf value[e]	%[f]
									0[c]	1.0	–
A	0.7	11.0	38.4	33.3	12.6	3.2	0.8	–	1	0.8	16.5
B	2.6	48.9	24.6	6.2	2.1	3.0	8.3	4.3	1	0.7	22.3
C	0.9	4.0	13.0	28.1	30.0	17.8	6.2	–	2	0.5	61.2
									3[d]	0.2	–
Totals[g]	2.1	11.4	16.7	24.8	23.4	14.8	5.1	1.1			

[a]Fraction of orange roughy wax esters.

[b]Determined by gas liquid chromatography.

[c]Saturated wax ester standard.

[d]Triunsaturated wax ester standard.

[e]Rf value of saturated wax esters = 1.0.

[f]Expressed as weight percentage of total wax esters.

[g]Ref. 2.

TABLE 2

Composition of Orange Roughy Wax Ester Fractions Isolated by Thin Layer Chromatography

Designation[a]	Band A		Band B		Band C	
	Acyl- (%)[b]	Alkyl- (%)	Acyl- (%)	Alkyl- (%)	Acyl- (%)	Alkyl- (%)
n-Saturated						
14:0	4.1	2.1	0.8	2.6	0.1	–
15:0	0.5	tr	0.2	1.1	–	–
16:0	6.3	44.1	2.1	48.5	0.5	1.6
17:0	0.2	3.4	1.1	1.9	0.2	–
18:0	2.5	18.7	0.5	17.0	0.2	–
20:0	–	3.5	–	2.1	–	–
22:0	–	1.4	–	0.2	–	–
24:0	–	3.5	–	1.4	–	–
26:0	–	3.2	–	0.9	–	1.3
28:0	–	2.8	–	1.0	–	2.6
n-Unsaturated						
14:1	tr[c]	–	0.2	–	0.4	–
15:1	0.3	–	0.1	–	0.4	–
16:1	1.4	1.1	10.4	1.1	9.7	0.7
17:1	tr	2.8	1.0	2.8	0.8	–
18:1	9.7	1.9	64.8	2.0	48.2	18.0
20:1	49.2	6.2	11.4	3.9	28.1	41.0
22:1	25.6	5.3	7.2	7.9	11.8	30.4
24:1	0.2	–	0.2	5.2	–	4.4

[a]Chain length: double bonds.

[b]Expressed as weight percentage of total fatty acids and fatty alcohols, respectively.

[c]Trace, less than 0.1%.

as it has been observed when mixtures of fatty methyl esters (5) and wax esters (6) have been separated by conventional TLC. The unexpected concentration of wax esters containing 44 and 46 carbon atoms in Band B may be related to their solubility in the solvent employed. By examining the individual acyl- and alkyl-wax ester components (Table 2), the main difference between esters in Bands A and B is in the chain-lengths of their corresponding acyl groups. The distribution of alkyl groups was similar in both fractions, and these appear to have little influence on the overall chain-length effects of these wax esters. The main components of Band C were of relatively longer chain-length than those of Band A.

The positions of double bonds in the derivatized individual monounsaturated acyl- and alkyl-moieties were established by GC-MS, and these findings (with comparisons) are shown in Table 3. Double bonds in the shorter chain-length fatty acids, 14:1, 16:1 and 18:1 are predominantly in the Δ9 position, whereas for 20:1, the Δ11 isomer was the major component. This Δ11 dominance extended into the 22:1 fraction, but substantial quantities of Δ13 (erucic acid) also were present. A wider range of positional isomers was noted in the 24:1 fraction. Even though Δ15 was the major isomer, appreciable quantities of Δ13 and Δ11 isomers also were available. In many animal systems Δ15 (nervonic acid) is an important part of the sphingomyelin content of nervous tissues (19).

The distribution pattern of double bond locations in the fatty alcohols over the 16:1 to 22:1 range resembled that observed for the corresponding acyl-fragments. However, this did not extend to the 24:1 fatty alcohol isomers. These differed markedly from their related fatty acids in that at least 92% of these isomers had the Δ15 structure.

DISCUSSION

Wax esters have an important role to play as part of the metabolic biological energy resources available to many forms of marine life dwelling at different ocean depths (20,21). Apart from representing the bulk of the body oil of some deep-water teleost fish (22) and their corresponding dietary sources of small zooplankton crustacea classed as copepods (21-24), wax esters also occur as a sizeable portion of the particulate lipid concentrates distributed in sea water (25).

Evidence for the incorporation of the primary source of dietary wax esters in fish is illustrated by matching (Table 3) the acyl-alkyl pattern of copepods with the individual acyl- and alkyl-wax ester components detected in the oils of mackerel and capelin (23,24). Although dietary wax esters are hydrolyzed initially in the fish gut (26), the released fatty acids (including the corresponding oxidized fatty alcohol products) are absorbed through the intestinal wall and, by means of de novo biosynthetic reactions, produce fatty alcohols which can be formally distributed as the acyl- and alkyl-moieties within the 16:1 to 22:1 range of isomers in Table 3 are closely related. Nevertheless, these procedures do not appear to hold for the C24 monoene isomers detected in orange roughy oil.

The data in Table 3 suggest that the predominant monounsaturated acids and alcohols of orange roughy oil up to 18:1 are produced primarily by Δ9-desaturation of the corresponding saturated components (27). After this, the expected chain elongation biosynthetic pathway (27) has yielded intermediate products of 20:1 Δ11, 22:1 Δ13 and 24:1 Δ15. Orange roughy also extended the Δ9-desaturase activities into the 20:0 area to provide a significant quantity (15%) of a 20:1 Δ9 isomer. This step initiated another series of related elongated products, 22:1 Δ11 and 24:1 Δ13.

The desaturase activities on fatty acids are not restricted to the Δ9-position alone (28).

TABLE 3

Comparison and Distribution of Wax Ester Monounsaturated Acyl- and Alkyl- Isomers Found in Orange Roughy Oil and Other Marine Species

Designation[a]	Isomer	Acyl-moieties (%)					Alkyl-moieties (%)				
		Orange[b] roughy	Mackerel[cd]	Capelin[cd]	Copepod[cd]	Sperm[be] whale	Orange[b] roughy	Mackerel[cd]	Capelin[cd]	Copepod[cd]	Sperm[be] whale
14:1	(ω5) Δ 9	100	–	–	–	70	–	–	–	–	–
16:1	(ω9) Δ 7	1	17	11	3	80	8	1	–	–	25
	(ω7) Δ 9	99	81	83	90	20	92	91	100	91	75
	(ω5) Δ11	–	3	6	8	–	–	8	–	9	–
18:1	(ω11) Δ 7	4	–	–	–	7	3	–	–	–	8
	(ω9) Δ 9	94	72	71	83	86	89	54	70	57	84
	(ω7) Δ11	2	23	21	13	7	8	39	30	3	8
	(ω5) Δ13	–	5	8	4	–	–	7	–	–	–
20:1	(ω11) Δ 9	15	7	6	7	50	4	3	–	6	20
	(ω9) Δ11	85	87	80	83	50	94	90	92	91	75
	(ω7) Δ13	–	5	12	10	–	2	6	7	3	5
	(ω5) Δ15	–	1	2	–	–	–	–	–	–	–
22:1	(ω13) Δ 9	8	92	85	86	17	2	84	89	91	–
	(ω11) Δ11	67				66	67				
	(ω9) Δ13	21	7	13	12	17	26	14	9	7	–
	(ω7) Δ15	4	1	2	2		5	2	2	2	–
24:1	(ω15) Δ 9	10	–	–	–	–	–	–	–	–	–
	(ω13) Δ11	21	–	–	–	–	2	–	–	–	–
	(ω11) Δ13	30	–	–	–	–	6	–	–	–	–
	(ω9) Δ15	39	–	–	–	–	92	–	–	–	–

[a]Chain length: double bonds.
[b]Determined by GC-MS.
[c]Determined by GC.
[d]Calculated from Refs. 23 and 24.
[e]Ref. 12.

Other alternative desaturation reactions appear to produce other monoenoic acid isomers and their related chain elongation products. Of all the extensive range of 24:1 desaturated acyl products that eventuated (Table 3), only one of these (24:1 Δ15) appeared to be modified to an alcohol.

Such variations of desaturase activities were noticed with the other marine species listed (Table 3) when both 18:1 Δ9 and 18:1 Δ11 acids and alcohols were identified (23,24). Because the proportion of these acyl- and alkyl-moieties are similar, this indicated the established de novo biosynthesis conditions related to reversible reactions between 16:0 acid and 16:0 alcohol (29,30) could be extended (31) to the other acyl-alkyl-isomers detected here. Exception seemed to apply to the 16:0 moieties of sperm oil when 16:1 Δ7 acid was reported as the main isomer (12). Nevertheless, the related sperm oil 16:1 alcohols with 16:1 Δ9 as the major isomer did fit into the general pattern for marine oils.

The application of derivatization techniques with GC-MS to determine the position of double bonds in monoenoic esters is well established (32,33). Their corresponding spectral fragmentation patterns to indicate the location of the double bonds was more specific for such structural determination than is reliance on GC data alone. The structures of the alkyl-acetates also were confirmed by comparing the MS of their oxidized (fatty acid) products with that of their related acyl-ester counterparts included with this investigation of orange roughy wax esters.

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Hypolipidemic Activity of the Surfactants Aminimides, and Their Effects on Lipid Metabolism of Rodents

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ABSTRACT

A series of short chain fatty acid derivatives of aminimides were shown to possess hypolipidemic activity in rats and mice. Most of the agents tested lowered both serum cholesterol and triglyceride levels by at least 30% in mice and were effective in hyperlipidemic induced mice. 1,1-Dimethyl-1-(2-hydroxypropyl)-amine mersitimide lowered serum cholesterol levels 41% and serum triglyceride levels 56% at 20 mg/kg/day I.P. after 16 days. The same agent was active orally when administered to rats with a 38% reduction in serum cholesterol and a 52% reduction in serum triglycerides after 14 days. The agents inhibited liver acetyl CoA synthetase, ATP dependent citrate lyase and phosphatidate phosphohydrolase activities in vitro and in vivo. Reduction of cholesterol, triglycerides, neutral lipids and phospholipid levels were noted in the livers of mice treated for 16 days. In rat studies, cholesterol, triglyceride and phospholipid levels were reduced in liver, small intestine and the feces after two weeks' dosing. The cholesterol content was reduced in the very low density lipoprotein (VLDL) and low density lipoprotein (LDL) fractions but elevated in the high density lipoproteins (HDL). Triglyceride levels were lowered in the VLDL, and neutral lipid levels were reduced in the chylomicron and VLDL fractions.

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INTRODUCTION

Previously, the cyclic and acyclic imides have been shown to possess hypolipidemic activity in mice and rats (1-5) at a dose of 20 mg/kg/day. When compared to clofibrate, the imides are more potent. Clofibrate, at 150 mg/kg I.P. in mice, lowers serum cholesterol levels from 12-15% and serum triglycerides by 25%. Reduced N-substituted cyclic and acyclic imides, including the indazolone series, retain good hypolipidemic activity in rodents (6). A series of short chain fatty acid derivatives of aminimides, a new class of industrial surfactants which have been tested recently for antimicrobial and antifungal activities (7-9), have a structure similar to the acyclic derivatives. Thus, it was decided to test these aminimides for potential hypolipidemic activity in rodents. Those results are now reported.

EXPERIMENTAL PROCEDURES

Sources of Compounds

The six compounds used for this study were prepared by Ashland Chemical Co. (Dublin, Ohio) and have been reported previously (7-9) (Table 1).

Antihyperlipidemic Screens in Normal Rodents

The aminimides were suspended in 1% carboxymethylcellulose-water, homogenized, and administered to CF_1 male mice (~25 g) intraperitoneally for 16 days. On days 9 and 16, blood was obtained by tail vein bleeding and the serum separated by centrifugation for 3 min. Sprague-Dawley rats (~300 g) were administered compound II at 20 mg/kg/day orally by intubation needle for 14 days. Blood samples were collected by tail vein bleeding on days 9 and 14. Control animals were administered 0.2 ml of 1% carboxymethylcellulose as vehicle and bled on the same days. All blood samples were collected between 8-9 a.m. Non-hemolyzed samples were used for the assays. The serum cholesterol levels were determined by a modification of the Liebermann-Burchard reaction (10). Serum also was collected on day 16 for mice and day 14 for rats, and the triglyceride content was determined using the Fisher, Hycell Triglyceride Test Kit.

Testing in Hyperlipidemic Mice

CF_1 male mice (~25 g) were placed on a commercial diet (U.S. Biochemical Corporation Basal Atherogenic diet) which contained butterfat (400 g), celufil (60 g), cholesterol (53 g), choline dihydrogen citrate (4 g), Wesson Oil (40 g), sodium cholate (20 g), sucrose (223 g), vitamin-free casein (200 g) and total vitamin supplement, for 10 days. After the cholesterol and triglyceride levels were assayed and observed to be elevated, the mice were administered test drugs at 20 mg/kg/day intraperitoneally for an

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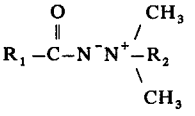
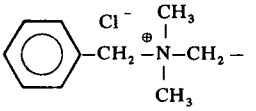
TABLE I

Structure of Aminimide Hypolipidemic Agents



Compound number	R_1	R_2
I	$CH_3(CH_2)_{10}-$	$-CH_2-CH(OH)-CH_3$
II	$CH_3(CH_2)_{12}-$	$-CH_2-CH(OH)-CH_3$
III	$CH_3(CH_2)_{14}-$	$-CH_2-CH(OH)-CH_3$
IV	$CH_3(CH_2)_{16}-$	$-CH_2-CH(OH)-CH_3$
V	$CH_2=C(CH_3)-$	$-CH_2-CH(OH)-(CH_2)_{11}CH_3$
VI		$-CH_2-CH(OH)-(CH_2)_{13}CH_3$

additional 14-day period. Serum cholesterol and triglyceride levels were measured after 12 days of administration of the drugs.

Enzymatic Studies

In vitro enzymatic studies were determined using 10% homogenates of CF_1 mouse liver with 50-200 μM of test drug. In vivo enzymatic studies were determined using 10% homogenates of liver from CF_1 male mice obtained after administering the agents for 16 days at a dose of 20 mg/kg/day intraperitoneally. The liver homogenates for both in vitro and in vivo studies were prepared in 0.25 M sucrose + 0.001 M (ethylenedinitrilo)tetraacetic acid, pH 7.2. Acetyl coenzyme A synthetase (11) and adenosine triphosphate dependent citrate lyase (12) activities were determined spectrophotometrically at 540 nm as the hydroxamate of acetyl coenzyme A formed after 30 min at 37 C. Mitochondrial citrate exchange was determined by the procedure of Robinson et al. (13,14), using ^{14}C-sodium bicarbonate (41 mCi/mmol) incorporated into mitochondria ^{14}C-citrate after isolating rat mitochondria (9000 g × 10 min) from the homogenates. The exchanges of the ^{14}C-citrate were determined after incubating the mitochondrial fraction, which was loaded with labeled citrate and test drugs for 10 min. Then the radioactivity was measured in the mitochondrial and supernatant fractions in Fisher Scintiverse fluid using a Packard Scintillation counter and expressed as a percentage of the total counts. Cholesterol side chain oxidation was determined by the method of Kritchevsky and Tepper (15) using (26-^{14}C)-cholesterol (50 mCi/mmol) and mitochondria isolated from rat liver homogenates. After 18 hr incubation at 37 C with test drugs, the generated $^{14}CO_2$ was trapped in the center well in {2-[2-(p-1,1,3,3-tetramethylbutylcresoxy)ethyoxy]ethyl}dimethylbenzylammonium hydroxide (Hyamine Hydroxide) and counted. 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMG coenzyme A reductase) was measured using 1-^{14}C-acetate (56 mCi/mmol) using a post-mitochondrial supernatant (9000 g × 20 min) incubated for 60 min at 37 C (16). The digitonide derivative of cholesterol was isolated and counted (17). Acetyl coenzyme A carboxylase activity was measured by the method of Greenspan and Lowenstein (18). Initially, the enzyme had to be polymerized for 30 min at 37 C, and then the assay mixture containing sodium ^{14}C-bicarbonate (41.0 mCi/mmol) was added and incubated for 30 min at 37 C with test drugs. Fatty acid synthetase activity was

determined by the method of Brady et al. (19) using [2-^{14}C]malonyl-coenzyme A (37.5 Ci/mmol), which was incorporated into newly synthesized fatty acids that were extracted with ether and counted. *sn*-Glycerol-3-phosphate acyl transferase activity was determined with glycerol-3-phosphate [L-2-^{3}H(N)] (7.1 Ci/mmol) and the microsomal fraction of the liver (20). The reaction was terminated after 20 min, and the lipids were extracted with chloroform:methanol (1:1) containing 1% conc. HCl and counted. Phosphatidate phosphohydrolase activity was measured as the inorganic phosphate released after 30 min from phosphatidic acid by the method of Mavis et al. (21). The released inorganic phosphate after development with ascorbic acid and ammonium molybdate was determined at 820 nm.

Liver Lipid Extraction

In CF_1 male mice that had been administered test drugs for 16 days, the livers were removed, and in Sprague-Dawley rats treated orally with compound II for 14 days, the liver, small intestine and 24-hr fecal samples were collected. A 10% homogenate in 0.25 M sucrose + 0.001 M (ethylenedinitrilo)tetraacetic acid was prepared for each tissue. An aliquot (2 ml) of the homogenate was extracted by the methods of Folch et al. (22) and Bligh and Dyer (23), and the number of mg of lipid weighed. The lipid was taken up in methylene chloride and the cholesterol level (4), triglyceride levels, (Bio-Dynamics/bmc Triglyceride Kit) neutral lipid content (24) and phospholipid content (25) were determined. Protein was determined by the method of Lowry et al. (26).

^{3}H-Cholesterol Distribution in Rats

Sprague-Dawley male rats (~350 g) were administered compound II orally for 14 days. On day 13, 10 μCi of ^{3}H-cholesterol was administered orally to rats, and feces were collected over the next 24 hr. Twenty-four hr after cholesterol administration, the major organs were excised and samples of blood, chyme and urine were obtained. Homogenates (10%) were prepared of the tissues which were combusted (Packard Tissue Oxidizer) and counted. Some tissue samples were plated on filter paper (Whatman #1), dried and digested for 24 hr in base at 40 C and counted. Resulted were expressed as dpm per total organ.

Plasma Lipoprotein Fractions

Sprague-Dawley male rats (~350 g) were administered compound II at 20 kg/day orally for 14 days. On day 14, blood was collected from the abdominal vein. Serum was separated from whole blood by centrifugation at 3500 rpm. Aliquots (3 ml) were separated by ultracentrifugation according to the methods of Havel et al. (27) as modified for normal rats (28) into the chylomicrons, VLDL, HDL and LDL. Each of the fractions was analyzed for cholesterol (4), triglyceride, neutral lipids (24), phospholipids (25) and protein levels. (26).

RESULTS

The aminimide derivatives proved to be effective hypolipidemic agents in normal mice, when administered intraperitoneally (Table 2). Serum cholesterol levels were lowered after 16 days by compounds I, II, III, IV and VI by at least 30%. All six compounds lowered serum triglyceride levels more than 30%; however, compound V afforded 43%, and compound II 56% suppression of triglyceride levels. 1,1 Dimethyl-1-(2-hydroxypropylamine meristimide) (II) after oral administration to rats, demonstrated, after 14 days, 38% reduction of serum cholesterol levels and 52% reduction of serum triglyceride levels.

In hyperlipidemic mice, serum cholesterol levels were elevated 183% (354 mg%) above normal control values (125 mg%) using a high lipid diet. Treatment with compound II for 12 days resulted in cholesterol levels being 23% (154 mg%) higher than the control value. The serum triglyceride levels in the hyperlipidemic mice were elevated 168% (376 mg/dl) above the control value (137 mg/dl). Treatment with compound II for 12 days resulted in values 15% (157 mg/dl) above control values.

Upon in vitro examination of the activities of regulatory enzymes from CF_1 mice involved in fatty acid, cholesterol and triglyceride de novo synthesis, it became evident that the aminimide derivatives had their major effects on liver acetyl CoA synthetase, ATP dependent citrate lyase and phosphatidate phosphohydrolase activities in vitro. The six derivatives essentially had no effect on mitochondrial citrate exchange, HMG CoA reduction, acetyl CoA carboxylase and fatty acid synthetase activities at 50, 100 and 200 μM concentrations in vitro. Compound II was the most effective agent in lowering ATP dependent citrate lyase activity maximally by 42% at 200 μM, with IV affording 25% inhibition at 200 μM. Compounds I and II suppressed acetyl CoA synthetase activity 28-29% at 200 μM. *sn*-Glycerol-3-phosphate acyl transferase activity was effectively inhibited ~35% by III and IV at 200 μM, while IV caused 59%, II 21% and I 19% inhibition. However, phosphatide

TABLE 2

The Effects of Aminimides on the Serum Cholesterol and Triglyceride Levels of CF_1 Male Mice (n=6) after Treatment at 20 mg/kg/Day Intraperitoneally for 16 Days, and Sprague-Dawley Rats after Oral Administration for 14 Days

Mouse (n=6) Compound number	Serum cholesterol Day 9 $\bar{X}$ ± SD (mg/dl)	Serum cholesterol Day 16 $\bar{X}$ ± SD (mg/dl)	Serum triglyceride Day 16 $\bar{X}$ ± SD (mg/dl)
I	83 ± 7[a]	79 ± 8[a]	78 ± 7[a]
II	82 ± 6[a]	74 ± 6[a]	60 ± 4[a]
III	125 ± 7	84 ± 6[a]	90 ± 5[a]
IV	85 ± 5[a]	84 ± 6[a]	77 ± 8[a]
V	112 ± 7	114 ± 9	92 ± 8[a]
VI	79 ± 6[a]	75 ± 5[a]	83 ± 5[a]
Clofibrate 160 mg/kg	109 ± 8	109 ± 6	102 ± 7
1% Carboxymethyl-cellulose	124 ± 7	125 ± 6	136 ± 8

Rat (n=6)	Serum cholesterol Day 9	Serum cholesterol Day 14	Serum triglyceride Day 9	Serum triglyceride Day 16
Compound II	60 ± 4	48 ± 3[a]	55 ± 6[a]	54 ± 4[a]
1% Carboxymethyl-cellulose	72 ± 5	78 ± 5	110 ± 9	112 ± 7

[a]$P < 0.001$.

phosphohydrolase activity was inhibited by all of the aminimides tested from 50-200 μM demonstrating a dose response curve. All of the agents afforded greater than 40% inhibition at 200 μM, with I resulting in 87%, II 54%, III 50%, IV 89% and V 42% inhibition. Cholesterol side chain oxidation was suppressed 40% by I and 35% by II at 100 μM concentration.

Examination of the same enzyme activities in vivo at 20 mg/kg/day after 16 days treatment of mice showed that HMG CoA reductase, fatty acid synthetase and *sn*-glycerol-3-phosphate acyl transferase were not altered after drug administration of any of the six derivatives at 20 mg/kg/day. Both ATP dependent citrate lyase and acetyl CoA synthetase activities were significantly inhibited. ATP dependent citrate lyase activity was suppressed more than 20% by all of the derivatives except IV. Acetyl CoA synthetase activity was inhibited 38-41% by compounds I-III, with IV affording 32%, V 27% and VI 21% inhibition. Acetyl CoA carboxylase activity was inhibited 13% by II and 11% by IV. Phosphatidate phosphohydrolase activity was inhibited by all six compounds, with I causing 79% inhibition, II 69%, IV 83% and V 44% inhibition (Table 3). The lipid content of the liver from the mice also demonstrated reductions (Table 4). Both cholesterol and triglyceride content were lowered by at least 35% by compounds I and II. Phospholipid contents were lowered 33% and 29% by I and II, respectively. Neutral lipids were affected only moderately, with 11-13% reductions after drug treatment.

After oral administration of compound II to rats for 14 days, there was a reduction of the lipid content of the liver, small intestine and feces; this was reflected in the cholesterol and triglyceride contents of each tissue. The phospholipid content of the liver was significantly reduced by compound II (Table 4). The serum lipoprotein fractions of rats also demonstrated changes in lipid content after drug administration, with a reduction of cholesterol content of VLDL (64%) and LDL (75%) but an increase of 114% of the cholesterol content of the HDL fraction. The triglyceride content was reduced 14% in the chylomicron fraction and 35% in the VLDL, whereas the LDL and HDL fractions were elevated 29-31% by drug treatment. Neutral lipids were reduced 52% in the chylomicron fraction and 73% in the VLDL fraction. Phospholipid levels were reduced 26% in the chylomicrons and elevated 26% in the HDL fraction (Table 5).

In rats, compound II had a small effect on body weight over the 14-day administration period. Normal rats over this period had a 12.5% increase in body weight, whereas the treated rats lost 6% of their body weight, i.e., an 18.5% difference between the two groups.

Daily food consumption over the same period was reduced 22% by compound II. The weights of the individual organs of the rats

TABLE 3

In Vivo Effects of Aminimides on the Activities of Mouse Liver Enzymes
Dosing for 16 Days Intraperitoneally

	Percent control	
(n=6)	ATP dependent citrate lyase activity mg citrate hydrolyzed/ gm wet tissue/20 min $\bar{X} \pm SD$	Acetyl CoA synthetase activity mg acetyl CoA formed/ gm wet tissue/20 min $\bar{X} \pm SD$
1% Carboxymethyl-cellulose	30.5 ± 2.1	28.5 ± 1.4
Treated		
I	21.9 ± 1.8[a]	17.7 ± 1.4[a]
II	23.5 ± 1.8[a]	16.8 ± 1.4[a]
III	22.8 ± 2.1[a]	17.4 ± 1.1[a]
IV	26.8 ± 2.4	19.4 ± 1.2[a]
V	24.1 ± 2.1[a]	20.8 ± 1.1[a]
VI	–	22.5 ± 1.4[a]
	Acetyl CoA carboxylase activity dpm/gm wet tissue/30 min $\bar{X} \pm SD$	Phosphatidate phosphohydrolase activity μg Pi released/gm wet tissue/15 min $\bar{X} \pm SD$
1% Carboxymethyl-cellulose	32010 ± 1918	16.72 ± 1.00
Treated		
I	30409 ± 1924	3.51 ± 0.33[a]
II	27849 ± 1600[b]	5.35 ± 0.51[a]
III	34891 ± 2241	5.52 ± 0.67[a]
IV	28489 ± 1921	2.84 ± 0.31[a]
V	35531 ± 1926	8.19 ± 0.49[a]
VI	–	9.36 ± 0.29[a]

[a] $P \leq 0.001$.
[b] $P \leq 0.010$.

treated with compound II were not markedly affected. Adrenal weights were not increased due to drug treatment, which does not suggest hypertrophy of the adrenal cortex as a compensatory stereogenesis mechanism due to low cholesterol levels in blood. The rat ^{3}H-cholesterol distribution study on day 14 showed that cholesterol was not being deposited in the major organs of the body, e.g. the heart (9887 total dpm for control) and spleen (12,250) demonstrated essentially no change in total dpm/organ. The brain (19,941 dpm) and large intestine (32,996 dpm) demonstrated minimum decreases of 13% and 14% respectively, whereas the lung (46,102 dpm) showed a 40% reduction, the liver (189,441 dpm) a 32%, the kidney (43,368 dpm) a 61% and small intestine (202,193 dpm) a 47% reduction in ^{3}H-content of cholesterol and its metabolite. The plasma level of ^{3}H-cholesterol was 43% less in treated rats (546 dpm/ml) when compared to control values (830 dpm/ml). High levels of cholesterol were observed in the stomach (72,795) and in the chyme (84,482) of the treated group compared to control values (56,564 and 59,803 dpm, respectively). The fecal content showed an actual decrease (from 85,006 to 67,391 dpms) in ^{3}H-cholesterol excretion after drug treatment, indicating there was no increase in cholesterol excretion via this route after drug treatment.

Data are expressed in Tables 3-4 as percent of control ± the standard deviation. The probable significant level (P) between each test group and the control group was determined by Student's "t" test.

DISCUSSION

This preliminary study to determine if short chain fatty acids of aminimides possess hypolipidemic activity in rodents was successful. Previously, the laboratory has shown that a series of cyclic imides (1-6) demonstrated significant reduction of serum cholesterol and triglyceride levels in rats and mice. The aminimide

TABLE 4

The Effects of Aminimides on the Lipid Content of CF_1 Mouse Liver after Treatment for 16 Days at 20 mg/kg/Day, Intraperitoneally and Sprague-Dawley Rat Liver, Small Intestine and Feces after Treatment for 14 Days at 20 mg/kg/Day Orally

CF_1 mouse liver Compound (n=6)	mg/lipid per 0.2 gm wet tissue extracted	Percent control ($\bar{X}$ ± SD), mg/gm wet tissue				
		Cholesterol	Triglycerides	Neutral lipids	Phospholipids (Pi)	Protein
1% Carboxymethylcellulose	19.0 ± 1.3	12.24 ± 0.73	4.77 ± 0.24	28.35 ± 1.98	4.39 ± 0.35	4.52 ± 0.32
I	14.8 ± 0.9[a]	7.46 ± 0.59[a]	2.48 ± 0.19[a]	21.83 ± 1.70[a]	2.94 ± 0.31[a]	4.43 ± 0.27
II	13.5 ± 1.1[a]	6.98 ± 0.58[a]	2.58 ± 0.22[a]	22.40 ± 1.68[a]	3.12 ± 0.26[a]	4.47 ± 0.36
III	13.8 ± 1.0[a]	9.67 ± 0.72[a]	3.53 ± 0.28[a]	26.93 ± 1.95	3.73 ± 0.38	4.61 ± 0.25
IV	16.5 ± 1.2	10.40 ± 0.61[b]	2.19 ± 0.17[a]	23.81 ± 2.27[b]	2.15 ± 0.18[a]	4.57 ± 0.34
V	18.6 ± 1.4	11.88 ± 0.75	4.92 ± 0.29	26.08 ± 1.99	4.34 ± 0.24	4.53 ± 0.37
VI	16.0 ± 1.2[b]	10.65 ± 0.85	4.67 ± 0.21	23.01 ± 1.93[b]	3.91 ± 0.22	4.43 ± 0.28
Rat liver						
Control	11.7 ± 0.9	24.03 ± 1.44	6.37 ± 0.38	44.11 ± 3.96	7.19 ± 0.70	4.51 ± 0.23
Compound II	8.5 ± 0.7[a]	15.85 ± 0.96[a]	3.69 ± 0.44[a]	42.30 ± 3.52	4.24 ± 0.51[a]	3.43 ± 0.36[a]
Rat small intestine						
Control	9.1 ± 0.7	7.82 ± 0.55	1.12 ± 0.08	6.98 ± 0.56	2.06 ± 0.19	42.0 ± 2.9
Compound II	6.9 ± 0.5[a]	6.18 ± 0.47[a]	0.77 ± 0.06[a]	6.07 ± 0.63	1.77 ± 0.12	39.9 ± 3.4
Rat feces						
Control	1.6 ± 0.1	28.47 ± 2.27	1.86 ± 0.13	33.94 ± 2.38	1.39 ± 0.11	6.99 ± 0.56
Compound II	1.4 ± 0.1[a]	23.06 ± 2.56	0.87 ± 0.09[a]	32.92 ± 2.72	1.26 ± 0.13	6.50 ± 0.42

[a] $p \leq 0.001$.
[b] $p \leq 0.010$.

TABLE 5

The Effect of Aminimides on the Lipid Content of Rat Serum Lipoprotein Fractions after 14 Days with Compound II, Orally at 20 mg/kg/Day

Serum lipoprotein fractions (μg/ml serum)	$\bar{X}$ ± SD				
	Cholesterol	Triglycerides	Neutral lipids	Phospholipids	Proteins
Chylomicron					
Control	337 ± 20	420 ± 21	67 ± 5	149 ± 10	184 ± 13
Compound II	276 ± 24[b]	361 ± 25[b]	32 ± 5[a]	110 ± 9[a]	167 ± 15
VLDL					
Control	190 ± 13	22 ± 2	98 ± 9	26 ± 2	50 ± 3
Compound II	65 ± 8[a]	14 ± 1[a]	26 ± 5[a]	26 ± 2	44 ± 4
LDL					
Control	210 ± 13	45 ± 3	10 ± 1	41 ± 3	122 ± 7
Compound II	52 ± 6[a]	58 ± 3[a]	9 ± 1	38 ± 4	125 ± 7
HDL					
Control	544 ± 43	27 ± 2	620 ± 49	153 ± 12	657 ± 39
Compound II	1164 ± 49[a]	35 ± 2[a]	694 ± 56	192 ± 11[a]	617 ± 33

[a] $p < 0.001$.
[b] $p < 0.010$.

series of analogues appears to be effective in the same dosage range as the cyclic imides in mice and rats, i.e., 20 mg/kg/day. This is a lower dose than required for the commercially available agent, clofibrate (27). Clofibrate, when given intraperitoneally at 150 mg/kg/day in mice, lowers serum lipid only marginally. The most effective agent of the aminimide series appears to be compound II, which lowers serum cholesterol levels 41% and serum triglyceride levels 56% on day 16 in mice. In rats, cholesterol levels were reduced 38% and serum triglycerides 52% after 14 days administration of compound II. It should be noted that the aminimides were active when administered either orally or intraperitoneally to rodents.

Other active agents were I and IV in the mouse screen. The aminimides were not only active in normal mice, but they also proved to be effective in induced hyperlipidemic mice, lowering the elevated cholesterol levels 56% from 354 to 154 mg and triglyceride levels 57% from 376 to 157 mg/dl. After 12 days, the lipid levels in the blood, after drug treatment, had not reached normal levels but were still elevated 23% for cholesterol and 15% for triglyceride. Compound II significantly lowered the lipid levels of the mice so that they could no longer be considered hyperlipidemic.

The aminimide derivatives suppressed the availability of acetyl CoA in the cytoplasm of the liver, both in vitro and in vivo. Acetyl CoA is a key intermediate required in the early synthesis of cholesterol and fatty acids. Furthermore, one of the regulatory enzymes of triglyceride synthesis, phosphatidate phosphohydrolase, was inhibited markedly by the aminimide short chain fatty acid derivatives. Lamb et al. (20) have demonstrated a correlation between inhibition of this enzyme and the lowering of serum triglycerides; in addition, similar observations have been made with the cyclic imides. The aminimides did not affect HMG CoA reductase activity or *sn*-glycerol-3-phosphate acyl transferase activity in vivo. These two enzymes are inhibited by clofibrate derivatives (27). Thus, the mode of action of the aminimide derivatives appears to be different from the hypolipidemic agent, clofibrate, but similar to the cyclic imides. The liver lipid levels were reduced by the potent aminimide agents. Compound II in rodents effectively lowered cholesterol, triglyceride and phospholipids, indicating that drug administration did not lead to lipid accumulations in tissues, which may be due to the lipids being removed from the blood and being deposited in the liver. In rats, a similar pattern of lipid content reduction was observed in the small intestine as well as the liver. The lower lipid content reflects the decrease in lipid synthesis produced by the inhibition of key regulator enzymes by the agents. ^{3}H-cholesterol distribution studies in rats demonstrated that ^{3}H-cholesterol was lowered in the major organs in the treated animals, when compared to the control animals. Higher content of ^{3}H-cholesterol was found in the stomach and in the chyme. Because the plasma ^{3}H level was lower in the treated animals after 24 hr, this may reflect a decrease in cholesterol absorption from the intestine afforded by the drug.

In rats, compound II reduced cholesterol content of the LDL fraction with a marked increase in cholesterol content in the HDL fraction. The LDL fraction supposedly is responsible for lipid deposition in the atherogenic plaques. The HDL fraction is responsible for removing cholesterol from the peripheral tissues which is carried to the liver for excretion. An increase in cholesterol content of HDL protects against atherosclerosis and related cardiovascular problems, e.g., myocardial infarction (28,29). Whereas changes in lipid content of rat lipoproteins cannot be directly related to man, these studies do demonstrate that aminimides are capable of modulating the lipid content of the serum lipoproteins in a favorable direction.

Further studies are required on the aminimides to establish their mode of action(s) as well as their effects of lipid distribution and excretion from the body. Additional SAR studies are required to elucidate the most effective hypolipidemic agent of this chemical group.

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The Distribution and Acyl Composition of Plasmalogens in Guinea Pig Heart

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ABSTRACT

In guinea pig heart homogenate, 34% of both choline and ethanolamine phosphoglycerides were in the form of plasmalogens (1-alkenyl, 2-acyl glycerophospholipid). Plasmalogens accounted for 39% of the choline phosphoglycerides and 36% of the ethanolamine phosphoglycerides in the mitochondrial fraction. Ethanolamine plasmalogen was the major ethanolamine phosphoglyceride (63%) in the guinea pig heart microsomal fraction. A high arachidonyl content was found in both diacyl and 1-alkenyl, 2-acyl glycerophosphoethanolamine. The C-2 fatty acyl profiles of the diacyl and 1-alkenyl, 2-acyl choline phosphoglycerides differed considerably from each other in the homogenate as well as in the subcellular fractions. Significant differences in the C-2 fatty acyl profiles also were observed in diacyl and 1-alkenyl, 2-acyl ethanolamine phosphoglycerides. Such differences suggest there is no direct metabolic relationship between the diacyl glycerophosphocholine (-ethanolamine) and its plasmalogen analog.
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INTRODUCTION

The widespread distribution of 1-alkenyl, 2-acyl glycerophospholipids (plasmalogens) in mammalian tissues is well documented (1). In most tissues the majority of plasmalogens are found as ethanolamine phosphoglycerides (2,3). However, high concentrations of choline and ethanolamine plasmalogens are present in some cardiac tissues (2,3). Although the metabolism of ethanolamine plasmalogens in some mammalian tissues has been reported (4-6), the metabolism of ethanolamine and choline plasmalogens in the heart is undefined. The physiological function of these phospholipids in cardiac or any tissue remains obscure.

Plasmalogens, like their diacyl analogues, possess a fatty acyl group esterified at the C-2 position of the glycerol backbone. However, the fatty acyl group of the plasmalogens are less susceptible to phospholipases than their diacyl counterparts (7,8). While the distribution and fatty acyl composition of diacylphospholipids and plasmalogens in subcellular fractions of some tissues have been studied (9-11), only limited information is available for the heart. In order to understand the metabolic, functional and structural roles of the cardiac plasmalogens, a detailed analysis of the subcellular distribution and molecular composition of these ether lipids is a definite prerequisite. In this communication, we report the distribution of choline and ethanolamine plasmalogens in guinea pig heart microsomal and mitochondrial fractions. The C-2 fatty acid composition of these plasmalogens and their diacyl analogues also are reported.

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MATERIALS AND METHODS

Materials

Phospholipase A_2 (*Crotalus adamenteus*), tris-HCl, sucrose, butylated hydroxytoluene were obtained from Sigma Chemical Co., St. Louis, Missouri. Thin layer chromatographic plates (sil-G25) were the product of Brinkman Instruments, Rexdale, Ontario. Fatty acid methyl ester standards and BF_3 were obtained from Supelco Inc., Bellefonte, Pennsylvania. Other chemicals and solvents were of reagent grade and were obtained from Fisher Scientific Co., Winnipeg, Manitoba. Male Albino guinea pigs, weighing 250-300 g, were obtained from Charles River (Ontario) and maintained on Purina Chow and tap water ad libitum in a light and temperature controlled room.

Subcellular Fractionation

Guinea pigs were decapitated and the hearts removed and placed on ice. Six hearts were used in each set of experiments; the hearts were washed, cut into pieces and homogenized in 0.25 M sucrose 10 mM tris-HCl and 2 mM EDTA at 4 C to yield a 10% (w/v) homogenate. Homogenization was performed two times with a Polytron (Brinkman model PT10-35) at a setting of 6 at 15 sec each. The homogenate was centrifuged for 4 min at 100 × g with a bench centrifuge at 4 C. The supernatant was decanted

and the pellet rehomogenized. This procedure was repeated twice and the combined supernatants together with the last homogenate were pooled. This final homogenate (H1), which contained 625 ± 48 mg protein (mean ± S.D. of 4 experiments), was centrifuged at 2,000 g for 10 min. The supernatant (H2), which contained 233 ± 5 mg protein, was centrifuged at 20,000 × g for 10 min, and the supernatant was recentrifuged at 20,000 × g once more for further sedimentation of any remaining mitochondrial fraction. The postmitochondrial fraction was centrifuged at 100,000 × g for 60 min to obtain the microsomal fraction. The mitochondria and microsomes were suspended in 0.25 M sucrose, 10 mM tris-HCl, pH 7.4.

Extraction of Lipids

Lipids were extracted from the homogenates by the method of Bligh and Dyer (12), and from the subcellular fractions by the method of Eichberg et al. (13). Butylated hydroxytoluene (0.25%) was added to all extracts. Phosphatidylcholine and phosphatidylethanolamine were isolated from the total lipid by thin layer chromatography (TLC) as previously described (14).

Analytical Methods

Protein was estimated by the method of Lowry et al. (15) with bovine serum albumin as the standard. Lipid phosphorus determinations were carried out by the method of Bartlett (16), and alkenyl content was measured by the method of Gottfried and Rapport (17).

Hydrolysis of Phospholipids

The alkenyl bond of plasmalogens was hydrolyzed by the procedure of Wells and Dittmer (18). Lysophospholipids derived from plasmalogens were separated using a solvent system of chloroform/methanol/water/acetic acid (70/30/4/2; v/v/v/v). After development, the plates were sprayed with dichlorofluorescein and the lipids were visualized under u.v. The lipid fractions on the thin layer chromatographic plates were removed and the lipids recovered by the method of Arvidson (19). Hydrolysis of the C-2 fatty acid of phospholipids was carried out by the method of Waku and Lands (20), with phospholipase A2 (*Crotalus adamenteus*).

Determination of Acyl Groups in Diacyl- and 1-alkenyl, 2-acyl Glycerophospholipids

Choline and ethanolamine phosphoglycerides, purified from total lipid extracts (14), were subjected to acid hydrolysis as described in the preceding paragraph. The products of the reaction, containing lysophospholipids derived from the plasmalogens and diacylphospholipids, were separated and recovered as described above. The diacylphospholipids extracted from the plates were then incubated with phospholipase A2. After completion of the hydrolysis, the fatty acids released were isolated from the reaction mixture by TLC with a solvent containing petroleum ether/diethyl ether/acetic acid (80/20/1). The TLC plates were sprayed with dichlorofluorescein and visualized with u.v. The fatty acids were extracted by the method of Arvidson (19), and methyl esters were formed in the presence of BF3. The methyl esters were analyzed by GLC as described previously (14).

Enzyme Assays

The cross contamination between mitochondrial and microsomal fractions was assessed by marker enzyme activities. Both fumurase (21) and succinate dehydrogenase (22) were used as marker enzymes for mitochondria, whereas glucose-6-phosphatase (23) and K^+ stimulated nitrophenyl phosphatase (24) were used as microsomal markers.

RESULTS

Mitochondrial and microsomal fractions were isolated from the guinea pig heart, and their purity was assessed by the activities of marker enzymes in each fraction. The results showed a 13% contamination of the microsomal fraction by mitochondrial enzymes and an 8% contamination of the mitochondria by microsomal enzymes. Further purification of the fractions by sucrose density centrifugation decreased the yield without any significant changes in cross contamination. Lipid cross contamination by the two fractions was estimated to be close to that computed from the marker enzymes because both subcellular fractions had similar ratios of lipid-P/mg protein (Table 1).

Lipids were extracted from guinea pig heart homogenates, mitochondria and microsomes. The proportion of choline and ethanolamine glycerophospholipids in these extracts was determined. Subsequently, the plasmalogen contents in these two phospholipids were analyzed; the results of these assays are summarized in Table 1. The values obtained for the mitochondria and microsomes could not be related quantitatively to that of the whole homogenate (H1), because the homogenate still contained a substantial amount of unbroken cells, nuclei and cell debris. The 2,000 × g centrifugation which we used to remove the nuclei materials also caused the removal of unbroken cells and other cell debris. Thus, the contribution by micro-

TABLE 1

Choline and Ethanolamine Glycerophospholipid Contents in Guinea Pig Heart Subcellular Fractions

	Lipid-P[a]	CPG[b,c]	% CPG as choline plasmalogen	EPG[b,c]	% EPG as ethanolamine plasmalogen
Homogenate	5.7 ± 0.8[d]	38.7 ± 4.2	(34.2 ± 0.9)	30.3 ± 2.5	(34.5 ± 1.7)
Microsomes	14.1 ± 0.6	41.3 ± 3.5	(36.6 ± 3.6)	31.9 ± 3.2	(62.8 ± 5.7)
Mitochondria	13.1 ± 0.7	41.8 ± 2.1	(39.3 ± 5.1)	27.8 ± 3.3	(36.1 ± 4.8)

[a]μg of lipid-P/mg protein.

[b]% of total lipid-P.

[c]CPG, choline phosphoglyceride; EPG, ethanolamine phosphoglyceride.

[d]The data represent mean ± standard deviation from 6 separate sets of experiments.

somes and mitochondria to the total lipid and protein content of the tissue from the homogenate could not be estimated. However, with respect to the supernatant obtained after the low speed centrifugation (H2), we observed that 32% of the total protein and 95% of the total lipid-P were recovered in the mitochondrial and microsomal fractions. Of this, 70% of the protein and 67% of the lipid were associated with the mitochondrial fraction, with the remainder found in the microsomal fraction.

Choline glycerophospholipids accounted for 38% of the total phospholipids compared to 30% for the ethanolamine phospholipids in the tissue homogenates. About 35% of both phospholipids were plasmalogens. The proportion of choline and ethanolamine phospholipids in the microsomal and mitochondrial fractions were similar, but their plasmalogen contents were different. In the mitochondria, only 39% of choline and 36% of ethanolamine glycerophospholipids were plasmalogens, whereas in the microsomes the majority of ethanolamine phospholipids (63%) were plasmalogens. Microsomal choline glycerophospholipid plasmalogen content (36%) is similar to that found in the mitochondrial fraction.

The C-2 fatty acid composition of choline and ethanolamine plasmalogens and their diacyl analogues was determined. The alkenyl group of the plasmalogen was removed by acid hydrolysis as described in Methods, and the acyl group of the resultant lysophospholipid was analyzed. To monitor the specificity of the hydrolytic procedures, a control experiment with 1-palmitoyl, 2-oleoyl glycerophosphocholine was used as substrate for the reaction. Subsequent analysis showed that this molecule was not affected by acid hydrolysis, which indicates that the acyl groups are not hydrolyzed by this treatment. The acyl group at the C-2 position of the diacyl phospholipid was hydrolyzed by phospholipase A2 (*Crotalus adamenteus*), and the fatty acids released were converted to methyl esters and analyzed by GLC. The positional specificity of the reaction was again monitored by the use of 1-palmitoyl, 2-oleoyl glycerophosphocholine as substrate. After the completion of the reaction, only 7% of the palmitoyl group from the C-1 position was hydrolyzed, which suggests that the reaction is highly specific.

The results obtained for the tissue homogenate are depicted in Table 2. The fatty acid composition of both the choline and ethanolamine plasmalogens were different from their diacyl analogues. Choline plasmalogens were more unsaturated than the corresponding diacyl analogs. The major fatty acids at the C-2 position of the diacyl-glycerophosphocholine were C18:1 and C18:2. In the choline plasmalogens these were C18:2 and C20:4. On the other hand, both ethanolamine phospholipids were found to have the same degree of unsaturation and had C20:4 as the major C-2 fatty acid. There was substantially more C20:4 in the diacyl than the plasmalogen which, in turn, had two times more C22:6 than the former. However, both ethanolamine lipids were more unsaturated than the choline plasmalogen.

Tables 3 and 4 show the results obtained for the guinea pig heart microsomes and mitochondria. It is clear that in both fractions, the ethanolamine glycerophospholipids were more unsaturated than their choline counterparts. The choline phospholipids had substantial quantities of oleic and linoleic groups in contrast to the ethanolamine phospholipids where the dominant C-2 fatty acyl residue was arachidonic acid. Fatty acyl profiles of diacyl glycerophosphocholine were similar between the mitochondrial and microsomal fractions, but the plasmalogen profiles in these fractions were very different. In microsomal choline plasmalogen,

TABLE 2

Acyl Composition at the C-2 Position of Choline Phosphoglycerides (CPG) and Ethanolamine Phosphoglycerides (EPG) from Guinea Pig Heart Homogenate

	CPG		EPG	
Acyl groups	Diacyl	Alkenyl-acyl	Diacyl	Alkenyl-acyl
16:0	17.7 ± 1.8[a]	8.4 ± 1.7	6.0 ± 1.6	6.2 ± 1.5
16:1	tr	tr	tr	tr
18:0	4.7 ± 1.3	2.1 ± 0.8	3.0 ± 0.5	2.6 ± 1.1
18:1	32.4 ± 1.3	14.4 ± 0.3	4.4 ± 0.8	4.6 ± 1.0
18:2	35.8 ± 2.7	34.4 ± 3.3	6.6 ± 1.0	8.1 ± 0.4
20:4 (n-6)	7.8 ± 1.7	28.6 ± 1.5	74.0 ± 1.3	50.3 ± 4.8
20:3 (n-3)	tr	tr	tr	tr
22:4 (n-6)	tr	3.2 ± 1.6	1.2 ± 0.7	5.7 ± 0.3
22:5 (n-6)	0.4 ± 0.1	1.4 ± 0.3	2.0 ± 0.8	5.6 ± 0.3
22:5 (n-3)	1.0 ± 0.5	6.4 ± 1.2	1.2 ± 0.6	13.0 ± 0.6
22:6 (n-3)	tr	1.1 ± 0.8	1.6 ± 0.5	3.5 ± 0.4

[a]The data are expressed as % of total acyl groups at the C-2 position, and are represented by mean ± standard deviation from 4 separate sets of experiments. tr = Trace amount of less than 1%.

TABLE 3

The Composition of Acyl Groups at the C-2 Position of Diacyl Choline Phosphoglycerides (CPG) and Diacyl Ethanolamine Phosphoglycerides (EPG) from Guinea Pig Heart Mitochondrial and Microsomal Fractions

	CPG		EPG	
Acyl groups	Microsomes	Mitochondria	Microsomes	Mitochondria
16:0	19.0 ± 1.56[a]	12.4 ± 1.0	0	0
16:1	tr	tr	tr	tr
18:0	5.5 ± 0.7	1.6 ± 0.3	0	0
18:1	34.0 ± 0.9	33.6 ± 1.2	7.2 ± 1.3	2.7 ± 0.7
18:2	34.5 ± 4.0	40.4 ± 1.8	14.3 ± 1.1	9.3 ± 0.9
20:4 (n-6)	7.3 ± 1.0	8.3 ± 0.7	61.8 ± 2.7	82.0 ± 3.4
20:5 (n-3)	tr	tr	2.8 ± 0.3	tr
22:4 (n-6)	tr	0.9 ± 0.2	2.2 ± 0.2	1.9 ± 0.4
22:5 (n-6)	tr	tr	1.7 ± 0.3	1.3 ± 0.3
22:5 (n-3)	tr	1.5 ± 0.4	7.2 ± 0.7	1.0 ± 0.2
22:6 (n-3)	tr	1.0 ± 0.2	2.8 ± 0.3	2.1 ± 0.2

[a]The data are expressed as % of total acyl content, and are represented by mean ± standard deviation from 4 separate sets of experiments.

18% of the C-2 fatty acids was palmitic acid, compared to 2% in the mitochondria. Conversely, choline plasmalogens in the mitochondria had more arachidonic acid than the microsomes. The situation was somewhat reversed with the ethanolamine glycerophospholipids. The fatty acid profile of the microsomal and mitochondrial ethanolamine plasmalogens were virtually identical, while the diacyl profiles showed substantial differences. Although both the ethanolamine plasmalogen and its diacyl analogue had arachidonic acid as the major fatty acid at the C-2 position, there were major differences between the two forms. The plasmalogens had substantially less C20:4 but more C22:5 and C22:6 than the diacyl glycerophosphoethanolamine.

DISCUSSION

Although most mammalian hearts have a large complement of phospholipid plasmalogens, there appears to be a species variation in the absolute amounts present (3,25). Our results on the diacyl and plasmalogen content of the choline and ethanolamine phospholipids isolated from guinea pig heart homogenates are in general agreement with published data (25). Upon

TABLE 4

The Composition of Acyl Groups at the C-2 Position of Choline Phosphoglyceride Plasmalogens and Ethanolamine Phosphoglyceride Plasmalogens from Guinea Pig Heart Mitochondrial and Microsomal Fractions

	CPG plasmalogens		EPG plasmalogens	
Acyl groups	Microsomes	Mitochondria	Microsomes	Mitochondria
16:0	18.6 ± 1.6[a]	2.2 ± 0.8	5.1 ± 1.2	3.3 ± 0.4
16:1	tr	tr	tr	tr
18:0	0	0	1.7 ± 0.3	1.9 ± 0.6
18:1	7.4 ± 1.2	14.3 ± 3.4	2.6 ± 0.4	2.4 ± 0.4
18:2	46.0 ± 3.2	37.8 ± 4.1	10.0 ± 0.6	10.7 ± 1.4
20:4 (n-6)	19.2 ± 1.8	30.3 ± 2.8	50.8 ± 2.8	50.4 ± 3.5
20:5 (n-3)	tr	tr	tr	2.5 ± 0.3
22:4 (n-6)	tr	1.5 ± 0.5	3.8 ± 0.5	2.7 ± 0.2
22:5 (n-6)	tr	1.1 ± 0.2	3.5 ± 0.3	3.5 ± 0.5
22:5 (n-3)	8.8 ± 1.4	9.2 ± 0.9	18.3 ± 0.6	17.1 ± 1.1
22:6 (n-3)	tr	2.6 ± 0.3	4.3 ± 1.1	5.5 ± 0.8

[a]The data are expressed as % of total acyl content, and are represented as mean ± standard deviation from 4 separate sets of experiments.

analysis of the C-2 fatty acyl contents, very little similarity was detected between the profiles obtained from the diacyl and the alk-1-enyl, 2-acyl moieties of these two phospholipids. Based on the structural similarity of the diacyl and alk-1-enyl, 2-acyl glycerophosphocholine in the bovine heart, Schmid and Takahashi (26) proposed that there was a direct metabolic relationship between these two analogues. The difference observed in the guinea pig heart suggests such a hypothesis may be valid only in selected mammalian species.

While the content and composition of choline and ethanolamine glycerophospholipids have been reported in several cardiac tissues, most of these studies made no attempt to differentiate the acyl contents of the plasmalogens from the diacyl phospholipids (27,28). At present, only the acyl contents of diacylphospholipids and plasmalogens in bovine heart have been well documented. Hence, comparison of the C-2 fatty acyl profile of guinea pig heart phospholipids were made with the bovine heart (26). Apart from the presence of the more saturated fatty acids (mainly C16:0), the C-2 fatty acyl profile of the diacyl glycerophosphocholine and -ethanolamine in the guinea pig heart was very similar to that of the bovine heart. However, major differences were observed among the fatty acids derived from choline and ethanolamine plasmalogens between the two species. There was substantially less C18:2 and more C22:5 in both choline and ethanolamine plasmalogens in the guinea pig heart than in the bovine heart, and such differences were more pronounced with the ethanolamine than the choline plasmalogens.

In order to complete our investigation on the acyl contents of the diacyl and alk-1-enyl, 2-acyl phospholipids in the guinea pig heart, subcellular fractions were prepared. The large majority of phospholipids in the postnuclei fraction (95%) was found in the mitochondrial and microsomal fractions. The cross contamination of each fraction is similar to the reported values (29). The difference in the C-2 acyl profiles between the diacyl and the alk-1-enyl, 2-acyl species of the choline and ethanolamine phospholipids in these subcellular fractions does not support a direct metabolic relationship between these molecular species. However, further evidence from turnover studies of the 2-acyl groups will be required to substantiate this postulation. The limited reports available on the fatty acid composition of the plasmalogens from subcellular fractions of mammalian hearts do not allow us to have any extensive comparison with other mammalian species. Recently, the molecular composition of canine myocardial sarcolemmal choline and ethanolamine phospholipid has been reported (3). Virtually all the C-2 acyl groups in phosphatidylethanolamine and 84% of the ethanolamine plasmalogens in this fraction were occupied by C20:4, but the choline phospholipids had C18:1 and C18:2 esterified in addition to C20:4. The C-2 acyl profiles of these phospholipids from the canine myocardial sarcolemma share some resemblance to the guinea pig heart microsomal fraction, with the exception of C22:5 or C22:6. These two acyl groups are present in significant quantities in the guinea pig microsomes, especially in the ethanolamine plasmalogens. Attempts to obtain sarcolemma fraction from the guinea pig heart

with the same degree of purity as reported in the canine heart were not successful. Thus, it is not known if the difference is due to species variation or the localization of some molecular species (containing C22:5 and C22:6) in the sarcoplasmic reticulum.

Ethanolamine glycerophospholipids of the guinea pig heart and of canine hearts (3) have a preponderance of C20:4 at the C-2 position. The concentration of C20:4 in the cardiac ethanolamine phospholipids contrasts sharply with results obtained for tissues such as the brain (9), where C22:6 was by far the major unsaturated species of ethanolamine glycerophospholipids. It has been postulated that the microsomal ethanolamine plasmalogens might represent a compartmentalized pool for the storage of prostaglandin precursors (30). However, in addition to the large quantities of C20:4 in guinea pig heart microsomal ethanolamine phospholipids, we also observed a large concentration of C20:4 in mitochondrial ethanolamine phospholipids. Although the role of ethanolamine plasmalogens in the production of prostaglandins is yet to be resolved, any studies to identify the source of the C20:4 will have to take cognizance of the presence of these two potential subcellular sources, and also to differentiate between the diacyl and plasmalogens as the immediate source within the compartment.

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Turnover of Phosphocholine and Phosphoethanolamine in Ether-Phospholipids of Krebs II Ascite Cells

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ABSTRACT

Krebs II ascite cells suspended in Eagle medium were incubated at 37 C for up to 6 hr in the presence of [^{3}H] glycerol or [^{32}P] orthophosphate. After extraction, their lipids were treated with guinea pig phospholipase A_1 under conditions where all diacyl-phospholipids (diacyl-PL) became hydrolyzed with 55% recovery of lyso-PL. Using a bidimensional thin layer chromatography (TLC) involving exposure to HCl fumes between the two runs, it then became possible to determine at once the specific radioactivity of the three subclasses (diacyl-, alkylacyl- and alkenylacyl-) present in choline glycerophospholipids (CGP) and ethanolamineglycerophospholipids (EGP). Compared to diacyl-PL, a lower de novo synthesis of ether subclasses was evidenced in both CGP and EGP by [^{3}H] glycerol incorporation. Although the same profile was obtained for CGP with [^{32}P] orthophosphate, the three EGP subclasses displayed in this case the same specific radioactivity.

These data indicate a higher turnover rate of the polar head group of ether-EGP compared to either-CGP. The simple methodology used in the present study might thus prove helpful in developing enzymatic studies dealing with the mechanism of this accelerated renewal.

Lipids, 20:699-703, 1985.

INTRODUCTION

We recently reported a high content of ether lipids of an ascitic strain, as measured by means of a new method allowing their rapid isolation from a total lipid extract (1). This method involves the separation of intact molecules of alkylacyl-subclasses and of lysophospholipids derived from alkenylacyl- and diacyl-subclasses, so that the polar head group of each type of compound is preserved. A method based on reverse phase high performance liquid chromatography (HPLC) coupled with chemical ionization mass spectrometry recently was described by Jungalwala et al. (2). However, such a technique requires complex equipment, so that our procedure still remains suitable to evaluate the turnover of the polar head group of the three phospholipid subclasses present in CGP and EGP. Since the work of Waku et al. (3), such studies rarely have been undertaken, because the current methods used for the separation of ether-phospholipids (ether-PL) require either the removal of the polar part (4,5) or a delicate fractionation procedure for analysis of hydrosoluble derivatives (3,6).

The present study deals with the determination of the turnover rate of the three subclasses (alkyl, alkenylacyl- and diacyl-) forming CGP and EGP. Our data indicate that the rate of renewal of the polar head group is the same in the three subclasses of EGP, whereas a lower turnover rate of phosphate is observed in ether-CGP compared to diacyl-CGP.

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MATERIALS AND METHODS

Chemicals

[1,3-^{3}H] glycerol (25 Ci/mmol) and [^{32}P] sodium orthophosphate were purchased from the Radiochemical Center (Amersham, United Kingdom). All the other products were from Merck (Darmstadt, Federal Republic of Germany) or Prolabo (Paris, France). Minimum Essential Medium of Eagle was supplied by Seromed (Lille, France). Guinea pig phospholipase A_1 was prepared as reported before (1). This corresponds to the cationic lipase isolated by DEAE-Sepharose chromatography (7).

Cell Isolation

Krebs II cells were grown in Swiss mice as already described (8). After washing in calcium-free Tyrode's buffer, ascitic cells were diluted in Eagle's medium buffered at pH 7.4 with 40 mM Hepes (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid). Final suspension was adjusted to 10^7 cells/ml, using a Nageotte counting cell.

Incorporation of Labeled Precursors

[^{3}H] glycerol (10 μCi) or [^{32}P] orthophosphate (40 μCi) were added to 4 ml of cell suspension. Both samples were incubated under gentle stirring for 6 hr. As Eagle's medium contains dihydrogenophosphate, the final specific radioactivity of [^{32}P] orthophosphate was 25 mCi/mmol. Measurements of pH and lactate dehydrogenase

activity through the whole incubation procedure indicated a good cell integrity without any change in medium pH.

Phospholipid Analysis

After incubation for different times, lipids were extracted immediately according to the method of Bligh and Dyer (9). They were then treated with guinea pig phospholipase A_1 under the following conditions: 400 nmol of total PL were dispersed in 1 ml of 0.2 M Tris-HCl (pH 7.4) containing 4.8 mM sodium deoxycholate (DOC), followed by sonication at 0 C for two 15-sec periods (Alcatel Sonifier, Bioblock Scientific, Strasbourg, France). Incubation was performed for 1 hr at 42 C in the presence of 1 IU of enzyme. After Bligh and Dyer extraction (9), the lipids from each sample were separated into two equal parts, which were deposited onto Silica Gel G plates, 0.25 mm thick (Merck, Darmstadt, Federal Republic of Germany). Phospholipid separation was performed by bidimensional thin layer chromatography with exposition of the plates to HCl fumes between the two runs (1). One chromatoplate was used to determine the radioactivity in the various spots, which was performed by liquid scintillation counting, using a Kontron Intertechnique spectrometer (SL 400) equipped with automatic quenching correction. The other one was used to measure phosphorus content of each spot (10). In both cases, sphingomyelin was determined for phosphorus content and was used as an internal standard.

RESULTS

Determination of Specific Radioactivity of Phospholipid Subclasses

As previously described (1), thin layer chromatography (performed as described above) of a lipid extract treated with phospholipase A_1 allowed us to identify seven spots: sphingomyelin; CGP and EGP, corresponding to alkylacyl-subclasses; lyso CGPe and lyso EGPe generated from diacyl-subclasses by phospholipase A_1 treatment; lyso CGPa and lyso EGPa formed upon acidic hydrolysis of alkenylacyl-subclasses. Under these conditions, phosphatidylserine, phosphatidylinositol and cardiolipin are completely destroyed and their lysoderivatives eliminated in the upper aqueous phase (1).

In our previous report (1), incubation conditions with phospholipase A_1 (pH 8, 2.4 mM DOC) promoted an almost total disappearance of lyso CGPe and lyso EGPe formed upon hydrolysis of diacyl-species. As shown in Figures 1C and 1D, increasing amounts of DOC (4.8 and 12 mM) impaired lyso PL degradation, especially upon lowering the pH to 7.4. This effect probably is due to an inhibition of acylmigration from the 2- to the 1-position of sn-glycerol, phospholipase A_1 being able to degrade 1-acyl-glycero PL at the same concentrations of detergent (11).

Under all conditions tested, degradation of diacyl-subclasses went to completion, since the amount of non-degraded CGP and EGP, which was shown to contain only ether-species (1), remained exactly the same (Figs. 1A and 1B).

The time course of phospholipid hydrolysis by phospholipase A_1 is shown in Figure 2. After 60 min, hydrolysis of diacyl-subclasses was achieved (Figs. 2A and 2B), whereas the amounts of lyso PL remaining on the plate represented only 55% of the diacyl-PL which had disappeared (Figs. 2C and 2D). In order to verify that lyso CGPe and lyso EGPe were representative of diacyl-CGP and diacyl-EGP, respectively, purified samples of CGP and EGP isolated from ^{32}P-labeled cells were incubated with phospholipase A_1. As shown in Table 1, the specific radioactivity of lyso-PL was essentially the same as that of sn-glycero-3-phosphocholine.

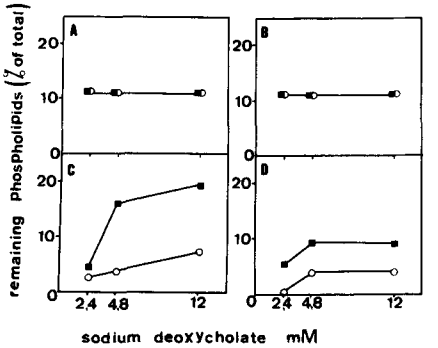

FIG. 1. Effect of two parameters (pH and sodium deoxycholate concentration) on the transformation of diacyl phospholipids into the corresponding lysoderivatives. An aliquot of total lipid extract from Krebs cells (400 nmol phospholipid) was evaporated under reduced pressure and dispersed by sonication in 1 ml of 0.2 M Tris-HCl buffer pH 7.4 (■) or pH 8.0 (○) containing various concentrations of sodium deoxycholate. Incubation was performed for 3 hr at 42 C under shaking in the presence of 1 IU of phospholipase A_1. Results are expressed as the remaining amounts of each phospholipid product after this treatment, compared to total phospholipids. A, choline-glycerophospholipids; B, ethanolamine glycerophospholipids; C, choline lysophospholipids generated by phospholipase A_1, and D, ethanolamine lysophospholipids generated by phospholipase A_1.

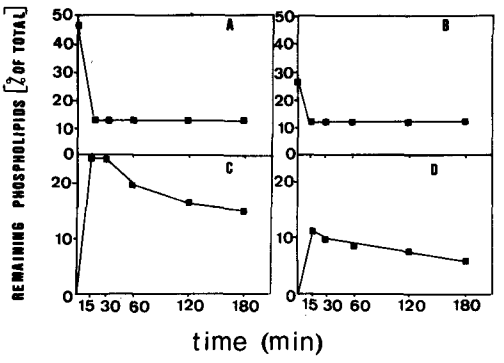

FIG. 2. Time dependence of lysophospholipid accumulation. Incubation parameters are described in Materials and Methods. Results are expressed as the remaining amounts of each phospholipid compared to total phospholipids of the cell. A, choline glycerophospholipids; B, ethanolamine glycerophospholipids; C, choline lysophospholipids generated by phospholipase A_1, and D, ethanolamine lysophospholipids generated by phospholipase A_1.

TABLE 1

Specific Radioactivities (dpm/nmol) of Lysophospholipids and of Hydrosoluble Glycerophospho-derivatives Generated by Phospholipase A_1 Treatment of ^{32}P-labeled Choline-and-Ethanolamine Glycerophospholipids

	Incubation time	
	15 min	60 min
lyso-CGPe	36.4 ± 0.5	39.7 ± 2.0
GPC	37.2 ± 2.8	36.6 ± 1.1
lyso-EGPe	39.9 ± 4.8	37.7 ± 5.4
GPE	37.9 ± 3.5	39.6 ± 2.2

Choline glycerophospholipids and ethanolamine glycerophospholipids, respectively, were purified from ^{32}P-labelled cells and incubated with phospholipase A_1 as described under Materials and Methods. After Bligh and Dyer extraction (9), lyso-choline glycerophospholipids (lyso CGPe) and lyso-ethanolamine-glycerophospholipids (lyso EGPe) were isolated by thin layer chromatography of the lower organic phase and determined for phosphorus and radioactivity content.

Specific radioactivity of sn-glycero-3-phosphocholine (GPC) and sn-glycero-3-phosphoethanolamine (GPE) was determined by measuring phosphorus and radioactivity in the upper aqueous phase.

Results are expressed in dpm/nmol and are means ±SD of three different experiments.

This also was true for lyso-EGPe and sn-glycero-3-phosphoethanolamine, indicating that lyso-PL reflected the total pool of diacyl-PL. So the conditions of incubation depicted under Materials and Methods were finally chosen and allowed us to perform accurate determination of specific radioactivity in the six phospholipid subclasses composing CGP and EGP.

Turnover Rate of the Polar Head Group of CGP and EGP Subclasses

Because preliminary experiments revealed that no net increase in phospholipid content was observed (as shown by cell numeration and lipid phosphorus determination), phospholipid turnover rate was estimated by measuring incorporation of [^3H] glycerol and [^{32}P] orthophosphate into the various phospholipid subclasses.

As shown in Figure 3, incorporation of the two precursors into PC proceeded in a similar way, diacyl-subclasses displaying in both cases a significantly higher specific radioactivity as compared to the two ether-PL subclasses. In contrast, whereas [^3H] glycerol labeling of diacyl-EGP was also greater than that of ether-EGP, the three EGP subclasses displayed almost equal specific radioactivities upon ^{32}P incorporation.

It is interesting to note that in each PL class and with both precursors, the specific radioactivity of alkylacyl-subclasses was never significantly higher than that of alkenylacyl-subclasses.

DISCUSSION

This work emphasizes the de novo synthesis of diacyl-, alkylacyl- and alkenylacyl-subclasses from PC and PE in a neoplastic cell. Reports in this field are essentially focused on diacyl- and alkenylacyl-species only, because they can be separated from each other easily by acidic treatment (12). The method used herein allows a rapid separation of intact molecules or lyso-derivatives from each subclass on a single chromatogram (1). Substrate specificity of the pancreatic phospholipase A_1 involved in our method has been investigated extensively in two recent reports (11,13).

The metabolism of the three phospholipid subclasses was studied under in vitro conditions. Compared to in vivo experiments performed on tumor bearing mice (3,14,15) or by intracranial injection of the precursor (6,16), our approach excludes the step of precursor migration to the target cells, which is a non-negligible parameter, especially for short incubation times (6).

Our data show a preferential utilization of the [1,3 ^3H] glycerol by diacyl-species, because this lipid precursor was poorly incorporated into ether subclasses. Such a difference could reflect simply a poor conversion of [1,3 ^3H]-sn-glycero-3-phosphate, the precursor of diacyl-PL, into

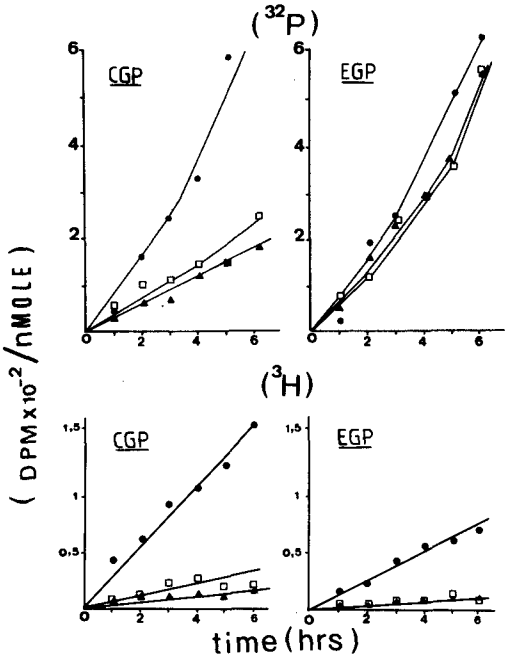

FIG. 3. Time course of [^{3}H] glycerol and [^{32}P] orthophosphate incorporation into choline glycerophospholipids (CGP) and ethanolamine glycerophospholipids (EGP) of Krebs ascite cells. Cells (4×10^{7}) were incubated at 37 C in Eagle medium containing [^{32}P]-orthophosphate or [^{3}H]-glycerol. After lipid extraction the three subclasses were separated as already reported (1). Results correspond to specific radioactivity in diacyl- (•), alkylacyl (▲) and alkenylacyl- (□) subclasses.

dihydroxyacetone phosphate, the precursor of ether-phospholipids, which could be better derived from glucose. A lower incorporation of [1,3 ^{3}H] glycerol into ether-PL compared to diacyl-PL might also come from the loss of one hydrogen atom from carbon 1 of dihydroxyacetone phosphate (17-19). Taking this fact into account, the specific radioactivity of ether-PL measured in the present study is probably 25% lower than the theoretical value, which would still be much less than the specific radioactivity of diacyl-PL.

It is noteworthy that in both CGP and EGP, the specific radioactivity of alkenylacyl-subclasses was superior or equal to that of alkylacyl-subclasses, which clearly have been shown to be the precursor of the former ones (20,21). This suggests a selective utilization of newly formed alkylacyl-species by the desaturase. Whether it corresponds to specific molecular species with a higher turnover rate remains to be determined.

In contrast, the use of ^{32}P revealed a marked difference between CGP and EGP, because [^{32}P]-orthophosphate was incorporated equally into the three EPG subclasses, whereas CGP revealed the same difference between diacyl- and ether-sublcasses as that previously observed with [^{3}H] glycerol. These data explain our previous observation that a higher labeling of EGP is obtained with ^{32}P than with [^{3}H] glycerol (22). They clearly indicate a higher turnover rate of the polar head group in alkylacyl- and alkenylacyl-EGP, as compared to ether-CGP.

Similar results were reported previously by Freysz et al. (6) in neuronal and glial cells. However, Waku et al. (3) observed an increased turnover of the polar head group in alkenylacyl-CGP and EGP, using Ehrlich ascites cells. Such a discrepancy might reflect differences in the origin of cells as well as in the incubation conditions.

The biochemical mechanism responsible for a rapid renewal of phosphoethanolamine in ether-PL still remains obscure. A similarly increased turnover triggered by specific agonists has been described largely for inositolphospholipids, but in this case, the phospholipase C responsible for phosphoinositide breakdown has been identified (23). As to the other glycero-PL, like CGP and EGP, the only phospholipase C so far described displays a lysosomal localization (24,25), although Edgar and Freysz (26) described a phospholipase C active against CGP in brain cytosol. But no specific affinity for ether-PL was reported before. However, Gunawan et al. (27) gave some evidence for a phospholipase C-like splitting of 1-alkenyl-sn-glycero-3-phosphoethanolamine in microsomal membranes from rat brain, which probably does not involve a lysophospholipase D (28). Finally, a reverse reaction catalyzed by diradylglycerol choline phosphotransferase has been described in some detail by Goracci et al. (29,30), so a similar pathway involving ether-EPG cannot be excluded.

Extensive studies of these possible pathways have been hampered so far by the difficulty to isolate ether-PL subclasses. These could be facilitated by applying the methodology described in the present paper. In this respect, it is interesting to note that Colard et al. (31) used the same experimental approach in studies dealing with transacylation reactions between platelet phospholipids.

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GSH-Dependent Inhibition of Lipid Peroxidation: Properties of a Potent Cytosolic System which Protects Cell Membranes

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ABSTRACT

Properties of a heat labile, nondialyzable cytosolic factor which prevents lipid peroxidation in membranous organelles are described. The factor is present in liver and other animal tissues, and its capacity to inhibit lipid peroxidation in membranes subjected to oxidative stress is greatly potentiated by glutathione (GSH), although GSH by itself has no inhibitory effect on lipid peroxidation. The data obtained thus far indicate that one or more sulfhydryl groups associated with the factor is required for the inhibition. The mechanism by which lipid peroxidation is inhibited must involve prevention of initiation of peroxidation in the membranes, presumably by a process requiring one or more sulfhydryl groups associated with the heat labile factor. The latter appears to be protected by GSH while the factor is exerting its inhibitory effect on lipid peroxidation. The factor is not one of the known GSH-dependent enzymes, and appears to be a potent and ubiquitous system for stabilizing cell membranes against oxidative damage.
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INTRODUCTION

Previous work by others and our laboratory has shown that liver cytosol contains a glutathione-dependent, heat labile factor which inhibits lipid peroxidation in biological membranes (1-3). The concensus of most of those investigations was that the heat labile factor was glutathione peroxidase. One report contained data indicating that the cytosolic factor was associated with the glutathione-S-transferases (2). However, later work in our laboratory demonstrated that the factor was neither glutathione peroxidase nor one of the glutathione-S-transferases (4). The inhibitory action of this factor was shown to require the presence of glutathione (4). If the liver cytosolic preparation is not dialyzed prior to testing for the inhibitory factor, the concentration of endogenous liver glutathione may still be sufficient to meet the requirement even if preparation of the liver homogenate resulted in a 1:10 dilution of the soluble contents of the liver. In fact, others have observed the inhibition of lipid peroxidation by liver cytosol without realizing that the inhibition was glutathione-dependent because undialyzed preparations of cytosol were employed (5,6). Thoroughly dialyzed liver cytosol alone will not inhibit lipid peroxidation unless the cytosol is added at high concentrations, but even low concentrations of cytosol will inhibit lipid peroxidation essentially completely in the presence of physiological concentrations of GSH (1-10 mM), as demonstrated in this report. The mechanism of the inhibition is unknown, but an earlier hypothesis, which proposed that the cytosol-GSH system reduces peroxide groups which may form in membrane phospholipids (5,6) was shown by our results to be unlikely (4). Our studies have demonstrated that there is no enzyme in liver cytosol of rodents capable of reducing peroxide functions in membrane phospholipids using GSH as a reductant. This includes both selenium-dependent and non-selenium-dependent glutathione peroxidases. Various reports imply that GSH peroxidases have such a function, but the evidence does not support that mechanism. GSH peroxidase can reduce free fatty acid hydroperoxides to free hydroxy fatty acids, but the enzyme will not reduce peroxide moieties in phospholipids (4). It is clear that the GSH-dependent inhibition of lipid peroxidation by the cytosolic factor occurs as a result of the prevention of lipid peroxidation even under conditions that should promote rapid peroxidation of membrane lipids. The factor appears to be one of the most potent antioxidant defenses in protecting membranes in animal tissue, since the concentrations of GSH which are most effective are those occurring in animal tissues. In this report we provide further details about this GSH-dependent cytosolic factor.

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MATERIALS AND METHODS

Preparation of the Cytosolic Factor

The rat liver cytosolic factor which inhibits both enzymic and nonenzymic lipid peroxidation was prepared according to the method of McCay et al. (1) from male Sprague-Dawley rats weighing ca. 200-250 g. The animals had been maintained from birth on a commercial laboratory animal ration. Rat liver microsomes were also prepared from these animals as described previously (5). The microsomes used were employed solely as a source of membrane lipids and were routinely heated to 100 C for 10 min, then cooled in ice and resuspended prior to use in the various test systems. The heat inactivation is necessary to prevent interference in the assay by the microsomal factor which inhibits lipid peroxidation in the presence of GSH, which has been described by Burk (8) and Reddy et al. (9). The latter factor has quite different properties than the cytosolic factor.

Assay for Peroxidation of Microsomal Membrane Lipids

In earlier work, it was demonstrated that the formation of thiobarbituric acid-reacting substances (primarily malondialdehyde) during lipid peroxidation was proportional to the loss of membrane polyunsaturated fatty acids (7). Hence, the thiobarbituric acid assay was used as a measure of the extent of lipid peroxidation. The reaction system contained heated rat liver microsomes (1.0 mg protein), 0.4 mM ADP, 12.0 μM Fe^{3+}, 0.66 mM ascorbate, various concentrations of GSH (or other thiol compounds which were tested) and different amounts of dialyzed rat liver cytosol. Potassium phosphate buffer (0.15 M, pH 7.5), was used to bring the final volume of the incubation system to 1.0 ml. The reaction systems were incubated for 30 min at 37 C. Malondialdehyde content was determined as thiobarbituric acid-reactive material according to the method of Bernheim et al. (10). A molar absorbancy of 1.56×10^5 was employed to calculate values as equivalents of malondialdehyde (11).

Inactivation of the Cytosolic Factor by Iodoacetate

The dialyzed rat liver cytosol was treated for 30 min with 0.25 mM 2-mercaptoethanol to reduce potential protein sulfhydryls. Then 5.0 mM iodoacetic acid (final concentration) was added to the preparation, and after 2 hr the preparation was dialyzed to remove unreacted iodoacetic acid. Cytosolic preparations treated in this manner were tested for their capacity to inhibit lipid peroxidation in the presence of GSH. Control cytosolic preparations were subjected to the same manipulations but without addition of iodoacetate.

Challenge Test for the Cytosolic Factor

In order to determine the duration that the GSH-dependent cytosolic factor could inhibit lipid peroxidation, large volume (20 ml) incubation systems were assembled as shown in Table 5. Because most of the peroxidizable lipids in the microsomes are consumed within the first 15 to 30 min, the thiobarbituric acid assay values reach a maximum in approximately the same period of time and remain relatively constant thereafter (18). At sequential times (0, 30, 180, 360 min), a 0.9 ml sample of each system was taken for analyses of thiobarbituric acid-reacting substances. This group of tubes was designated the prechallenge set. A duplicate 0.9 ml volume from each system was placed in another set of tubes, to which an additional 0.1 ml of the microsomal suspension was added and incubation at 37 C for another 30 min. These tubes were then also assayed for thiobarbituric acid-reacting substances (postchallenge set). For proper control, 0.1 ml of the microsomal suspension was also added to the prechallenge set after the reaction was terminated with trichloroacetic acid. Our experience has shown that the later addition of more microsomes provides the system with a fresh supply of peroxidizable lipid and the added membranes are peroxidized during a subsequent 30 min incubation period. The difference between the respective values of the thiobarbituric acid assays of the prechallenge and postchallenge sets of samples was a measure of the peroxidation of the added increment of microsomes. This technique demonstrated that the reaction system is still capable of promoting lipid peroxidation of added membranes up to 6.5 hr after initial assembly. Therefore, this procedure was used to determine if the GSH-dependent cytosolic factor was still capable of protecting a new addition of membranes added as long as six hr after the prechallenge system was assembled.

Lipid Conjugated Diene Determination

The conjugated diene content of the lipid fraction of the microsomes was determined on incubation systems which had been proportionally scaled up to a volume of 10 ml. After the systems were assembled, they were incubated for 15 min at 37 C. The systems were then centrifuged at 105,000 $\times$ g for 60 min. The microsomal pellets were removed and resuspended in 2.0 ml of 0.15 M potassium phosphate buffer, pH 7.5. Total lipids were extracted from the

resuspended microsomes by the addition of 20 volumes of chloroform-methanol (2:1, v/v) according to the method of Folch et al. (12). The lipids were recovered by solvent evaporation and dissolved in cyclohexane to a final concentration of 1 mg/ml. Scans of absorbance of these total lipid extracts were made from 200-400 nm. Any increment in conjugated diene content was determined by subtracting the absorbance at 300 nm from the absorbance at 234 nm.

Oxygen Consumption

The oxygen consumption studies were performed using a Clark type B oxygen electrode. The total volume of the systems used in these studies was 2.0 ml. The systems were assembled as described above except initiation of lipid peroxidation by the addition of ascorbic acid was not performed until after a period of 4 min to allow for temperature equilibration in the sample chamber. At the end of the equilibration period, ascorbic acid was added to initiate the reaction. O_2 consumption was followed for 10 min at 37 C.

RESULTS

Nonspecificity of the Requirement for GSH

Table 1 shows the results of experiments in which GSH was replaced by another sulfhydryl or disulfide compound in the lipid peroxidation assay system. 2-Mercaptoethanol, at the various concentrations shown, was as effective as GSH in the inhibition of lipid peroxidation by the cytosolic factor and, like GSH, caused no inhibition of lipid peroxidation when added without cytosol. When dithiothreitol was substituted for GSH, substantial inhibition of lipid peroxidation was observed at lower concentrations of this dithiol than with either GSH or 2-mercaptoethanol. The data suggest that the number of sulfhydryl groups added to the system may determine the extent of inhibition. Substituting GSH with cysteine also resulted in inhibition similar to that obtained with GSH. It is apparent from these results that the requirement for GSH for the inhibition of lipid peroxidation by liver cytosol is not a specific one. The effectiveness of the various sulfhydryl compounds in the system does not appear obviously related to their redox potentials.

Effect of Iodoacetate on the Inhibition of Lipid Peroxidation by the Cytosolic Factor

One possibility for the function of a required sulfhydryl compound to obtain sustained inhibition of lipid peroxidation is that the cytosolic factor may have one or more sulfhydryl groups required for the inhibition. To test this possibility, the cytosolic preparation was treated with iodoacetic acid, dialyzed, and tested for its capacity to inhibit lipid peroxidation (Table 2). The data show that the inhibitory activity is abolished by such treatment. Activity could not be regained by increasing the GSH concentration to 100 mM. In a separate experiment, the rat liver microsomes used in the assays were treated with iodoacetic acid to indicate whether a sulfhydryl group associated with these particles might also be required to obtain inhibition of lipid peroxidation. Lipid peroxidation in iodoacetate-treated microsomes was just as effectively inhibited by GSH + cytosol as peroxidation in systems containing microsomes not treated with iodoacetate (data not shown).

Effect of the GSH-Dependent Cytosolic Factor on Oxygen Consumption

Table 3 shows that O_2 consumption associated with lipid peroxidation is markedly inhibited by the GSH-dependent cytosolic factor. Addition of GSH alone to the peroxidation system, however, actually enhanced the extent of oxygen uptake under the conditions described (Table 3). If the cytosolic factor functions by reducing peroxide moieties on the phospholipids of membranes as hypothesized (15-17), a significant amount of O_2 consumption should be seen over control values during the course of the reaction. The data show that the rate of O_2 consumption in a system in which the cytosolic factor is preventing lipid peroxidation in microsomes exhibits very little O_2 uptake over that of the control system where no peroxidizing environment exists.

Effect of the GSH-Dependent Cytosolic Factor on Diene Conjugation

If the cytosolic factor functioned by a mechanism that resulted in reduction of lipid radicals ($L^{\bullet}$) formed by hydrogen abstraction, thereby preventing reaction of the lipid radicals with O_2, production of conjugated dienes in the lipid would still be expected because double bond migration accompanies the abstraction process. Since maximum peroxide content in this system develops within 15 min (18), the content of conjugated dienes in the lipid fraction should be maximum by 15 min also. Systems in which lipid peroxidation was suppressed by the GSH-dependent cytosolic factor for 15 min were subjected to total lipid extraction by the Folch procedure. The diene conjugate content of the lipid extracts was assayed and it was observed that the cytosolic factor in the presence of GSH

TABLE 1

Efficacy of Various Thiol Compounds for Substituting GSH in the Inhibition of Lipid Peroxidation by the Cytosolic Factor

Incubation systems	nmol MDA/mg protein ± S.D.
1. Δmicr + ADP-Fe^{3+}	2.36 ± 1.70
2. Δmicr + ADP-Fe^{3+} + AA	35.65 ± 7.83
3. Δmicr + ADP-Fe^{3+} + AA + CS	30.12 ± 9.93
4. Δmicr + ADP-Fe^{3+} + AA + CS + thiol	(see below)
5. Δmicr + ADP-Fe^{3+} + AA + thiol	(see below)

Thiol	Concentration (mM)	Cytosol added	No cytosol added
		nmol MDA/mg protein ± S.D.	
Glutathione	10.0	6.05 ± 3.03	39.19 ± 6.94
	5.0	6.46 ± 3.39	38.38 ± 7.25
	1.0	11.96 ± 4.72	36.76 ± 7.55
	0.5	17.24 ± 8.16	34.88 ± 10.22
	0.1	34.59 ± 9.11	35.52 ± 7.23
Oxidized glutathione	10.0	27.94 ± 9.67	31.90 ± 7.92
	5.0	32.12 ± 8.45	35.08 ± 8.86
	1.0	36.03 ± 10.70	34.19 ± 8.08
	0.5	36.60 ± 9.67	35.27 ± 10.75
	0.1	36.43 ± 9.91	37.16 ± 8.43
Cysteine	10.0	6.16 ± 3.87	11.18 ± 2.95
	5.0	7.14 ± 3.96	16.72 ± 2.40
	1.0	10.90 ± 6.44	27.87 ± 3.75
	0.5	16.30 ± 7.67	28.01 ± 6.54
	0.1	31.71 ± 11.18	34.52 ± 6.42
2-Mercaptoethanol	10.0	4.98 ± 2.50	39.87 ± 10.33
	5.0	5.83 ± 2.94	39.70 ± 10.14
	1.0	10.14 ± 5.33	38.77 ± 11.44
	0.5	17.73 ± 12.78	37.60 ± 9.85
	0.1	31.62 ± 9.80	37.68 ± 9.34
Dithiothreitol	10.0	3.61 ± 1.07	15.56 ± 14.55
	5.0	3.77 ± 1.07	18.94 ± 18.24
	1.0	4.11 ± 1.34	31.84 ± 11.03
	0.5	4.75 ± 1.63	34.82 ± 11.59
	0.1	28.49 ± 12.88	36.87 ± 9.63

All incubation systems consisted of the following unless otherwise indicated: ca. 1 mg heated rat liver microsomal protein (Δmicr) per ml reaction system, 0.4 mM ADP, 12.0 μM Fe^{3+}, 0.66 mM ascorbic acid (AA), and ca. 12 mg rat liver cytosolic protein (CS) per ml reaction system. Each thiol compound was tested by its addition to systems 4 and 5 at the concentrations indicated below. Reaction systems were incubated for 30 min at 37 C after which the thiobarbituric acid assay value (expressed as equivalents of malondialdehyde [MDA]) was determined (10).

totally suppressed the formation of conjugated dienes (Table 4). GSH alone had no effect, but the cytosolic factor alone caused a small but significant inhibition of diene conjugation. This experiment along with the O_2 consumption studies demonstrates that the GSH-dependent cytosolic factor must function by preventing the initiating hydrogen abstraction process (i.e., lipid radical formation), which in turn would avert the subsequent peroxidative loss of polyunsaturated fatty acids in biological membranes that would otherwise result from such abstraction.

Experiments Which Challenge the Capacity of the Cytosolic Factor to Inhibit Lipid Peroxidation

Another series of experiments was performed to determine how well the GSH-dependent cytosolic factor could sustain the inhibition of lipid peroxidation over longer periods. The results indicated that the cytosolic factor is capable of protecting biological membranes from peroxidative attack for extended periods. Nonenzymic peroxidation systems were assembled as described in Table 5. As stated in the Materials and Methods section, duplicate samples from each system were removed at the times indi-

TABLE 2

Inactivation of the Cytosolic Inhibitor of Lipid Peroxidation by Iodoacetate

Incubation system	nmol MDA/mg of microsomal protein ± S.D.
Δmicr + ADP-Fe^{3+}	3.03 ± 1.64
Δmicr + ADP-Fe^{3+} + AA	37.54 ± 2.48
Δmicr + ADP-Fe^{3+} + AA + GSH (10 mM)	43.23 ± 1.38
Δmicr + ADP-Fe^{3+} + AA + GSH (5 mM)	41.91 ± 1.02
Δmicr + ADP-Fe^{3+} + AA + GSH (1 mM)	40.78 ± 1.82
Δmicr + ADP-Fe^{3+} + AA + GSH (10 mM) + CS	7.45 ± 5.23
Δmicr + ADP-Fe^{3+} + AA + GSH (5 mM) + CS	10.20 ± 8.69
Δmicr + ADP-Fe^{3+} + AA + GSH (1 mM) + CS	17.10 ± 16.61
Δmicr + ADP-Fe^{3+} + AA + GSH (10 mM) + CS*[a]	35.51 ± 8.59
Δmicr + ADP-Fe^{3+} + AA + GSH (5 mM) + CS*	37.41 ± 3.73
Δmicr + ADP-Fe^{3+} + AA + GSH (1 mM) + CS*	36.47 ± 5.10
Δmicr + ADP-Fe^{3+} + AA + CS	34.06 ± 7.02
Δmicr + ADP-Fe^{3+} + AA + CS*	37.68 ± 6.10

The conditions of the experiment are described in the Materials and Methods section. The concentrations of the system components are as described as in Table 1 except GSH. The final concentrations of GSH are indicated where it was present. Addition of 0.4 ml of the cytosol preparation (CS) was made to systems requiring it. All experiments were performed a minimum of three times.

[a]CS* indicates addition of iodoacetate-treated cytosol.

TABLE 3

Inhibition of Oxygen Consumption in a Peroxidizing System by the GSH-Dependent Cytosolic Factor

Incubation systems	Oxygen uptake ± S.D. (nmol O_2 consumed min/mg protein)
Δmicr + ADP-Fe^{3+}	2.64 ± 1.35
Δmicr + ADP-Fe^{3+} + AA	31.61 ± 4.01
Δmicr + ADP-Fe^{3+} + AA + GSH	42.86 ± 8.93
Δmicr + ADP-Fe^{3+} + AA + GSH + CS	11.12 ± 2.18
Δmicr + ADP-Fe^{3+} + AA + CS	28.06 ± 10.00

The systems were prepared as described in Materials and Methods and Table 1. The GSH final concentration, where indicated, was 10 mM. A basal rate of O_2 consumption was obtained for 4 min after temperature equilibration at 37 C prior to the addition of ascorbic acid. Ascorbic acid (0.66 mM) was added to initiate the peroxidation reaction, followed for an additional 10 min using a Gilson oxygraph equipped with a Clark-type electrode. The total volume of all systems was adjusted to 2.0 ml by the appropriate addition of 0.15 M phosphate buffer, pH 7.5.

TABLE 4

Influence of the Cytosolic Factor and GSH on Lipid Diene Conjugation in Microsomes Subjected to Peroxidizing Conditions

Incubation systems	Absorbance ± S.D.[a]
Δmicr + ADP-Fe^{3+} + AA	0.667 ± 0.33
Δmicr + ADP-Fe^{3+} + AA + GSH	0.616 ± 0.36
Δmicr + ADP-Fe^{3+} + AA + GSH + CS	0.047 ± 0.04
Δmicr + ADP-Fe^{3+} + AA + CS	0.504 ± 0.10
Δmicr + ADP-Fe^{3+} + GSH + CS	0.038 ± 0.06

Microsomal lipid was extracted, dissolved in cyclohexane (1 mg/ml) and scanned 400-200 nm. Conditions of the incubation were the same as described in Table 1 except only a 15 min incubation at 37 C was performed and the GSH final concentration was 10 mM.

[a]Measured by subtracting the absorbance at 300 nm from the absorbance at 234 nm.

cated. One of the samples was analyzed for thiobarbituric acid-reacting substances (as malondialdehyde equivalents) for that particular time period, while the other sample was "challenged" by the addition of more microsomal membrane substrate. Prior studies in this and other laboratories have shown that the limiting factor in this reaction system is the availability in the microsomes of peroxidizable fatty acids, which essentially are depleted within 30-45 min after peroxidation is initiated (7,19). The results of the challenge experiments are shown in Table 5. Supplementing the standard incubation system (microsomes + ADP-Fe^{3+} + ascorbate) with additional microsomes after 30 min or as long as six hr after composing the original reaction system results in renewed peroxidation activity. (See challenge data for systems 2 and 3 at 30, 180 and 360 min in Table 5.)

The following results become immediately apparent:

(a) The cytosolic factor in the presence of 1.0 mM GSH resists the challenge (i.e., inhibits

TABLE 5

Composition of Reaction Systems Employed to Determine the Duration of the Capacity of the Cytosolic Factor to Inhibit Microsomal Lipid Peroxidation

Incubation time (min)	System number	Additions to system (final concentrations)		TBA values[a] malondialdehyde equivalents)	
		GSH (mM)	Cytosol (ml/ml of reaction system	Prechallenge	Postchallenge
0	1[a]	–	–	0.78	
	2	–	–	1.01	
	3	10.0	–	0.98	
	4	10.0	0.6	1.03	
	5	10.0	0.4	1.05	
	6	10.0	0.2	0.96	
	7	1.0	–	0.98	
	8	1.0	0.6	1.03	
	9	1.0	0.4	0.98	
	10	1.0	0.2	0.87	
	11	–	0.6	1.05	
	12	–	0.4	0.90	
	13	–	0.2	0.94	
30	1[a]	–	–	2.24	4.48
	2	–	–	25.58	59.58
	3	10.0	–	27.78	76.61
	4	10.0	0.6	2.44	2.28
	5	10.0	0.4	2.71	2.33
	6	10.0	0.2	3.99	4.52
	7	1.0	–	25.38	68.77
	8	1.0	0.6	3.00	3.32
	9	1.0	0.4	3.16	3.81
	10	1.0	0.2	5.22	8.09
	11	–	0.6	7.48	14.69
	12	–	0.4	16.20	47.94
	13	–	0.2	21.35	60.93
180	1[a]	–	–	9.83	12.03
	2	–	–	50.85	71.90
	3	10.0	–	57.57	102.37
	4	10.0	0.6	5.15	4.23
	5	10.0	0.4	4.75	4.39
	6	10.0	0.2	6.83	7.28
	7	1.0	–	46.82	81.31
	8	1.0	0.6	7.44	5.89
	9	1.0	0.4	7.46	7.39
	10	1.0	0.2	13.91	48.83
	11	–	0.6	14.07	12.05
	12	–	0.4	40.59	64.96
	13	–	0.2	43.01	68.99
360	1[a]	–	–	14.29	17.29
	2	–	–	60.03	56.22
	3	10.0	–	83.78	118.72
	4	10.0	0.6	8.53	8.09
	5	10.0	0.4	9.81	8.69
	6	10.0	0.2	11.81	11.11
	7	1.0	–	77.73	83.33
	8	1.0	0.6	12.30	11.02
	9	1.0	0.4	12.48	11.65
	10	1.0	0.2	22.56	35.32
	11	–	0.6	21.46	20.41
	12	–	0.4	59.36	71.01
	13	–	0.2	61.82	75.04

The basic incubation system contained 0.66 mM ascorbic acid, 0.4 mM ADP, 12.0 μM Fe^{3+}, and ca. 0.5 mg/ml heated rat liver microsomal protein. The final volume was brought up to 20 ml with 0.15 M potassium phosphate buffer, pH 7.4. At the end of the incubation time, two 0.9 ml aliquots of each of the systems were taken. An additional 0.5 mg of microsomal protein was added to each sample. One was immediately assayed for thiobarbituric acid-reacting substances (prechallenge value); the other was incubated for another 30 min at 37 C, and then subjected to the TBA test (postchallenge TBA value).

[a]System 1 did not contain ascorbate and was a control to determine that microsomes peroxidize very little in this system unless both ADP-Fe^{3+} and ascorbate are present.

lipid peroxidation) for at least 6.5 hr, providing sufficient cytosol was added. (See challenge data for systems 8 and 9 at 30, 180 and 360 min in Table 5.) However, at the lowest cytosol concentration tested in the presence of 1.0 mM GSH, the ability to resist the challenge was lost by 60 min (system 10).

(b) The highest concentrations of cytosol tested without the presence of GSH (system 11) also resisted the peroxidation challenge for the entire 6.5 hr period of the experiments. The lesser amounts of cytosol tested without GSH were not capable of resisting the peroxidation challenge test even for 30 min (systems 12 and 13). The results indicate that the concentration of cytosol is critical to the duration of its membrane stabilizing activity, i.e., its ability to inhibit lipid peroxidation under conditions where peroxidation should be occurring. However, other experiments show that the effectiveness of even the smallest amount of the cytosolic factor can be enhanced by raising the final concentration of GSH in the system from 1.0 mM to 10.0 mM (compare system 10 with system 6). The experiments demonstrate that GSH has the property of preserving the inhibitory activity of the cytosolic factor and that this property is particularly apparent when the concentration of cytosol in the system is relatively low. Because of the variable extent to which different preparations of microsomes undergo peroxidation at each of the several time intervals used, the data shown in Table 5 is for a single experiment, but the experiment has been performed four times and the pattern of results has been identical in every experiment.

DISCUSSION

In the initial studies on this inhibitory factor, we and others presumed that the inhibition of lipid peroxidation was caused by glutatione peroxidase (1.20). However, during the development of the purification process, it became clear that the factor was neither glutathione peroxidase nor the non-selenium-containing glutathione peroxidase associated with the glutatione-S-transferases (4). Its mechanism of action, although unknown, does not involve any reduction of peroxide groups in peroxidized membrane lipids, since no hydroxy fatty acids were formed when microsomes containing lipid peroxides were incubated in cytosol which contained GSH (1). It is still an enigma how a soluble protein together with GSH, both in the aqueous phase, can inhibit lipid peroxidation that would have occurred in the hydrophobic portion of the membrane. Because cytosol (at higher concentrations) can inhibit lipid peroxidation by itself, but not nearly as effectively as in the presence of GSH, and because the cytosolic factor requires its own -SH group (or groups) to show any inhibitory activity, it is possible that the factor is a protein containing one or more sulfhydryl groups that have an avid affinity for certain reactive forms of oxygen. An explanation for the shorter duration of protection provided by lower concentrations of cytosol may be that the protective thiol(s) are depleted by the inhibition process but may be regenerated if GSH is present. As proposed by Svingen et al., the ADP-Fe^{3+} chelate, when reduced by ascorbate, apparently forms a complex with molecular oxygen (21). In a hydrophobic environment, such a complex may possess hydrogen abstracting properties. Access of the nucleotide complex to the membrane polyunsaturated fatty acyl groups may be gained through an association with proteins in the lipid bilayer of membranes. Reaction of the thiol group(s) of the cytosolic factor with the reactive ADP-Fe^{3+}-oxygen complex could reduce the latter, producing a thiyl radical that is reduced by GSH in a reaction catalyzed by the protein itself. Without GSH, the thiol groups could function only once, and in the absence of GSH, the factor apparently undergoes a gradual inactivation while exerting its inhibitory activity. However, in the presence of GSH or some other small thiol compound, the factor may function for an extended period, as the results described above have shown.

A likely product of the reduction of the ADP-Fe^{3+}-oxygen complex would be H_2O_2, which would promptly be converted to H_2O by the glutathione peroxidase present in abundance in rat liver cytosol. This concept fits the results obtained in a number of ways. No conjugated dienes were formed in the membrane lipid in the inhibited system, indicating that H atom abstraction was suppressed by the cytosolic factor + GSH. Oxygen consumption was also suppressed by the cytosolic factor, but still was measurably more (11.12 nmoles/min) than the control (2.64 nmoles/min). (The control is the same system except no ascorbic acid is added to initiate lipid peroxidation [Table 3].) Ascorbate oxidation alone does not account for the oxygen differential. This oxygen differential could well be the result of the reduction by the cytosolic factor of a reactive form of O_2 associated with the ADP-Fe^{3+} chelate, thus preventing an oxidative attack on membrane lipids. In the inhibited state of the reaction system, GSH is slowly oxidized at a rate comparable to the O_2 consumed, after correcting for the autoxidation of GSH in the control system. This interpreta-

tion accounts for the results obtained, but proof of this role for the cytosolic factor would require, among other parameters, measurements of the oxidation of its thiol groups when incubated in the system in the absence and presence of GSH. Such an interpretation also would explain the inhibitory activity of the cytosol alone at high concentrations as well as the observation that it is GSH-dependent at low concentrations. Purification of the factor, now nearing completion in this laboratory, is necessary for such measurements. Progress to data indicates it is a single protein with a relative molecular mass of ca. 15,000 daltons.

These results demonstrate that the cytosolic fractions of all tissues tested thus far contain a protein which, in the presence of GSH, is extraordinarily potent in protecting biological membranes from iron-catalyzed lipid peroxidation. Failure of such a protective system may explain in part the cellular damage which results from the depletion of GSH in animal tissues. Although there are many thiol-containing proteins in liver cytosol, only this particular protein exhibits the properties described above. In addition, since the evidence upon which the factor is presumed to be a thiol protein is based on the results of the iodoacetate treatment, it is possible that the inhibitory effect of the latter may be due to some other effect. The findings verify that small thiols other than GSH can substitute for the latter in this system (13,14), but GSH is the only small thiol present in animal tissues in sufficient amounts to provide maximum protective action against lipid peroxidation.

It is conceivable that the GSH-dependent cytosolic factor possesses some type of free radical-quenching property and can enter and leave biological membranes easily, thereby providing the means for scavenging radicals within the membranes while being regenerated by GSH in the soluble fraction. However, there is presently no direct evidence to support this concept.

It appears likely that the suppression of lipid peroxidation by the cytosolic factor, and the preservation of this activity by GSH, plays an important role in maintaining membrane integrity in animal tissues. Strong support for this concept is drawn from a variety of observations which indicate that depletion of glutathione in tissues leads to oxidative damage to membranes (22-28).

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METHODS

An Improved Assay for Cholesterol 7α-Hydroxylase Activity Using Phospholipid Liposome Solubilized Substrate[1]

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ABSTRACT

A persistent problem in measurement of cholesterol 7α-hydroxylase (7α-OHase) activity by isotope incorporation has been solubilization of cholesterol substrate. Solubilization with Tween 20, for example, resulted in a 75% reduction in 7α-OHase activity after a 60 min incubation of substrate with microsomes. Incorporation of cholesterol substrate into small, unilamellar phospholipid vesicles (liposomes) prevented this effect, resulting in a 50% increase in activity over the same 60 min incubation at optimal concentrations. Using cholesterol in liposomes as substrate, standard assay conditions were determined to be: preparation of liposomes with 180 μM cholesterol substrate and 0.5 mg phospholipid/assay; incubation of these liposomes with 0.5 mg microsomal protein at 37 C for 60 min; addition of a NADPH generating system to start the reaction, and incubation at 37 C for 30 min before stopping the reaction and determining the amount of 7α-hydroxycholesterol formed. In addition to preventing the detergent-related inhibition of the enzyme, liposome-solubilized substrate also reduced the variation among replicates from a coefficient of 45% with Tween 20 to 4.2% with phospholipid. This method provides a sensitive and reliable alternative to methods which require more sophisticated equipment and allows total control of substrate concentration in a form readily accessible to the enzyme.
Lipids 20:712-718, 1985.

INTRODUCTION

The microsomal enzyme cholesterol 7α-hydroxylase (7α-OHase) catalyzes the first and rate limiting step in the conversion of cholesterol to bile acids (1,2). This mixed function oxidase requires molecular O_2, cytochrome P450 and NADPH cytochrome P450 reductase (3-5). Recently, it has been proposed that 7α-OHase is modulated on a short-term basis by phosphorylation/dephosphorylation (6,7), a mode of regulation that may be coupled to the short-term regulation of other microsomal enzymes of hepatic cholesterol metabolism (8).

Methods presently available for the assay of 7α-OHase activity include a double isotope derivative technique (9), gas-liquid chromatography coupled to mass spectrometry (10,11) and isotope incorporation into radioactive 7α-hydroxycholesterol (12). The first two methods require special instrumentation, limiting their utility as routine assays. They also utilize endogenous cholesterol as the sole source of substrate, not accounting for differences in substrate concentration due to experimental treatment (e.g., diet modification) or allowing adjustment to saturation of the enzyme with substrate. These factors make the isotope incorporation technique the most practical means of assaying 7α-OHase activity.

Several problems remain with the isotope incorporation assay. Activity may be underestimated due to incomplete equilibration of exogenous cholesterol with the endogenous pool to which the enzyme has access. Extraction of microsomes with cold organic solvent removes endogenous cholesterol (13-15) but does not assure availability of exogenous substrate to the enzyme and could result in alteration of the delicate membrane association of subunits necessary for the regulation of 7α-OHase activity. Acetone-extracted microsomes display only 10-20% of the activity routinely observed in fresh microsomes and appear to have lost phosphatase activity (15). A second problem involves solubilization of substrate with non-ionic detergents such as Tween 20. These detergents have been shown to inhibit 7α-OHase activity (16).

These problems led us to examine a detergent-free solubilization system in which the cholesterol substrate was incorporated into small, unilamellar vesicles (liposomes).

MATERIALS AND METHODS

Chemicals

[4-^{14}C]Cholesterol (Amersham Corp., Arlington Heights, Illinois; 58.4 mCi/m mole) was purified by thin layer chromatography (TLC)

[1] This work was reported in part at the American Society of Biological Chemists Meeting, St. Louis, Missouri, June 1984.

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on 250 micron silica gel G plates developed in ethyl acetate:hexane (8:2, v/v) (17) using standards for cholesterol and 7α- and 7β-hydroxycholesterol. The radioactive cholesterol band was removed, eluted with anhydrous $CHCl_3$, filtered and diluted to 1-2 μCi/ml in anhydrous $CHCl_3$. Unlabeled cholesterol (Supelco, Bellefonte, Pennsylvania) was checked for purity as above and dissolved in anhydrous benzene. Both were stored at -20 C. A mixture of 7α- and 7β-hydroxycholesterol used as standard for TLC was prepared by the reduction of 7-ketocholesterol (Steraloids, Wilton, New Hampshire) with $NaBH_4$. Isomers were extracted with ether:benzene (1:1, v/v), washed with water, dried over anhydrous Na_2SO_4 and diluted to a concentration of 10 mg steroid/ml $CHCl_3$. Asolectin (95% mixed soy phospholipid; Associated Concentrates, Woodside, New York) was purified by the method of Kagawa and Racker (18) and dried under N_2. The dry phospholipid film was pulverized and stored desiccated at room temperature in the dark. Purified phospholipids (phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine) were obtained from Sigma Chemical Co., St. Louis, Missouri.

Animals

Male Wistar rats were housed in a room illuminated from 4 p.m. to 4 a.m. and given a commercial diet (Wayne Lab Blox, Continental Grain Co., Chicago, Illinois) and deionized water ad libitum. Rats were killed by decapitation at 10 a.m., the diurnal maximum in 7α-OHase activity (19).

Microsomal Preparation

Rat livers were placed in ice-cold, 300 mM sucrose solution immediately after excision. All subsequent steps were performed at 0-4 C. Livers were homogenized in 2.0 ml/g liver wet weight TEDKSF (250 mM sucrose, 40 mM Tris-HCl, 1 mM EDTA, 5 mM dithiothreitol [DTT], 50 mM KCl and 50 mM KF; pH 7.4) (20). The homogenate was centrifuged at 10,000 g for 10 min and the pellet was discarded. The supernatant was centrifuged at 105,000 g for 90 min and the resulting microsomal pellet was suspended in 1.0 ml/g liver wet weight TEDKF (40 mM Tris-HCl, 1 mM EDTA, 5 mM DTT, 50 mM KDl and 50 mM KF; pH 7.4) (20) and assayed for protein concentration (21). Microsomal suspensions were stored in liquid N_2.

Substrate Preparation

Cholesterol (0.1 μmole/assay [180 μM] or as indicated below) was combined with [4-^{14}C] cholesterol (0.1 μCi/assay) and either Tween 20 (TW, 1.5 mg/assay), mixed soy phospholipid (asolectin, 0.5 mg/assay or as indicated below), purified phospholipid (0.5 mg/assay), or a combination of TW and asolectin. Solvent was removed under a N_2 stream and the mixture was suspended in TEDKF (0.1 ml/assay). TW-solubilized substrate was suspended by thorough mixing (Vortex Genie, Scientific Industries, Inc., Bohemia, New York) and brief sonication (1-3 min) while substrates solubilized by the asolectin or a combination of asolectin and TW were placed in a bath sonicator until the solution became opalescent (5-15 min), an indication of formation of small unilamellar vesicles (liposomes) (22).

Cholesterol 7α-Hydroxylase Assay

Stage 1. Microsomal protein, 0.5 mg in 200 μl TEDKF, was added to 100 μl of the cholesterol substrate and incubated at 37 C for 60 min or times indicated below, taking care to prevent exposure to light to prevent nonenzymatic oxidation.

Stage 2. Assays were initiated by the addition of 250 μl of a NADPH-generating system consisting of 4.5 mM $MgCl_2$, 1.25 mM $NADP^+$, 2.5 mM glucose-6-phosphate and 5 units of glucose-6-phosphate dehydrogenase in 70 mM phosphate buffer (pH 7.4). Incubation at 37 C was continued for an additional 30 min, again preventing exposure to light. Assays were terminated by addition of 7.5 ml $CHCl_3$:MeOH (2:1, v/v) and thorough mixing.

Extraction and separation of products. After addition and thorough mixing of one ml of water, phases were allowed to separate at 4 C for one hr. The $CHCl_3$ phase was removed and placed in a 15 ml conical glass stoppered tube and the aqueous phase was again extracted with 3 ml of $CHCl_3$. The combined organic phases were evaporated under N_2. The residue was dissolved in 50 μl of $CHCl_3$, and a 20 μl portion was subjected to TLC on 250 micron silica gel G plates developed in ethyl acetate:hexane (8:2, v/v) using 7α- and 7β-hydroxycholesterol and cholesterol as standards. Areas corresponding to standards were visualized by I_2 vapor and, after allowing I_2 to sublime and solvents to evaporate overnight, 7α-hydroxycholesterol and cholesterol were removed and assayed for radioactivity by liquid scintillation spectrometry (LS1800; Beckman Instruments, Irvine, California). Enzyme activity was calculated as the amount of cholesterol substrate converted to 7α-hydroxycholesterol, as determined by the percent of radioactive cholesterol recovered as 7α-hydroxycholesterol and expressed as picomoles 7α-hydroxycholesterol formed per minute per milligram of microsomal protein (pmoles/min mg).

TABLE 1

Modification of Cholesterol 7α-Hydroxylase Activity Using Substrate Solubilized with Tween 20 Alone and Tween-Asolectin Combinations

Substrate solubilized with:[a]		Specific activity of 7α-OHase after stage 1 incubation	
Tween 20	Asolectin	0 min	60 min
mg/assay	mg/assay	pmoles/min mg	
0.5	0	33.1 ± 5.3[b]	24.0 ± 1.7
1.5	0	21.5 ± 2.7	5.9 ± 3.0
5.0	0	2.5 ± 2.7	2.2 ± 2.0
1.5	0	22.7 ± 2.6	10.5 ± 2.5
1.5	0.5	24.5 ± 1.6	30.8 ± 2.7
1.5	1.0	18.5 ± 2.4	24.6 ± 2.2
1.5	2.0	6.5 ± 0.3	15.0 ± 1.9
1.5	3.0	5.8 ± 0.7	27.3 ± 6.9
1.5	5.0	9.4 ± 1.7	14.9 ± 1.5

[a]Cholesterol (180 μM) solubilized by indicated amounts of Tween 20 alone or a combination of Tween 20 and asolectin as listed, and incubated with microsomes at 37 C for time indicated (stage 1).

[b]Mean ± standard deviation, n = 3.

RESULTS

Tween and Tween-Asolectin Solubilization

When TW was used to solubilize cholesterol substrate, a reduction from 18.7 ± 5.0 pmoles/min mg to 6.6 ± 3.3 pmoles/min mg (n = 15) occurred as a result of a 60 min stage 1 incubation. This inhibition was increased by addition of increasing amounts of TW for solubilization of the substrate during stage 1 (Table 1). In addition, at the highest concentration of TW used, the activity of 7α-OHase was inhibited without stage 1 incubation.

Combining asolectin with TW for substrate solubilization negated the inhibition observed with TW solubilization during stage 1 incubation (Table 1). Increasing concentrations of asolectin with constant TW concentration resulted in reduced 7α-OHase activity, but activity was increased by stage 1 incubation at all concentrations of asolectin (26 to 371%).

Asolectin Solubilization

The effect of increasing concentrations of asolectin used in the preparation of liposome-solubilized substrate compared with TW-solubilized substrate is shown in Table 2. When no stage 1 incubation was employed, asolectin-solubilized substrate resulted in significantly higher 7α-OHase activity than with TW alone. As the amount of asolectin used to solubilize substrate was increased, 7α-OHase activity remained unchanged through 0.5 mg/assay and declined above that concentration, both with and without stage 1 incubation. This effect was also observed when the cholesterol substrate concentration was 500 μM.

TABLE 2

Effect of Asolectin Concentration and Preincubation on Cholesterol 7α-Hydroxylase Activity

	Specific activity of 7α-OHase after stage 1 incubation	
Asolectin[a]	0 min	60 min
mg/assay	pmoles/min mg	
0[b]	19.1 ± 0.4[c]	5.7 ± 0.4
0.2	62.5 ± 1.5	110.6 ± 5.5
0.3	78.4 ± 3.0	116.4 ± 6.6
0.4	73.7 ± 2.1	111.9 ± 1.4
0.5	75.5 ± 4.0	113.3 ± 1.3
1.0	66.9 ± 2.6	87.6 ± 0.4
2.0	53.5 ± 2.9	57.0 ± 2.4
3.0	48.1 ± 3.0	23.0 ± 2.5
5.0	39.9 ± 8.0	5.0 ± 0.7

[a]Cholesterol (180 μM) solubilized with indicated amount of asolectin as liposomes and incubated with microsomes at 37 C for time indicated (stage 1).

[b]Cholesterol (180 μM) solubilized with 1.5 mg/assay Tween 20.

[c]Mean ± standard deviation, n = 3.

Optimization of Assay Parameters for Asolectin-Solubilized Substrate

Using the optimum asolectin concentration observed in the above experiments (0.5 mg/assay), the effects of substrate concentration and stage 1 incubation time on 7α-OHase activity are shown in Figures 1 and 2. Three prepara-

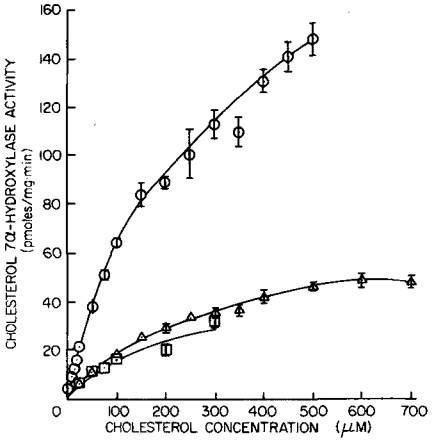

FIG. 1. Effect of substrate concentration on cholesterol 7α-hydroxylase activity. Cholesterol solubilized with 0.5 mg/assay asolectin and incubated (stage 1) with microsomal suspension for 60 min at 37 C. Stage 2 incubation time of 30 min at 37 C. ⊙ = microsomal preparation A; ⊡ = microsomal preparation B; △ = microsomal preparation C.

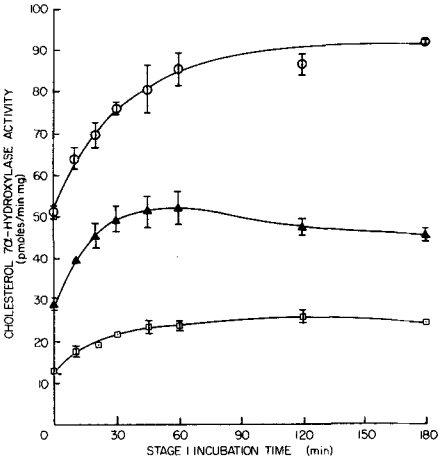

FIG. 2. Effect of stage 1 incubation time on cholesterol 7α-hydroxylase activity. Cholesterol solubilized with 0.5 mg/assay asolectin and incubated (stage 1) with microsomal suspension for time indicated at 37 C. ⊙ = microsomal preparation A, 180 μM cholesterol; ⊡ = microsomal preparation B, 180 μM cholesterol; ▲ = microsomal preparation C, 500 μM cholesterol.

tions of microsomes, each from a single rat liver, were used to demonstrate these effects.

In Figure 1, three substrate concentration ranges were employed, and in all three cases a nearly linear increase in the activity of 7α-OHase was observed through 50 μM. Activity continued to increase to a concentration of approximately 600 μM where the activity appeared to reach a plateau. A K_m of 290 ± 29 μM for the cholesterol substrate was calculated from these data.

Figure 2 depicts the effects of stage 1 incubation time on 7α-OHase activity at 180 and 500 μM cholesterol concentration. At 180 μM substrate concentration, activity increased with increasing stage 1 incubation time through 60 min. At the higher substrate concentration (500 μM), maximum activity was achieved at 45 min stage 1 incubation.

Activity of 7α-OHase increased in direct proportion to the amount of microsomal protein added to the incubation through 0.5 mg/assay, but activity decreased when levels above 1.0 mg/assay were added (Fig. 3).

Stage 2 incubation time also influenced the rate of conversion of cholesterol substrate to 7α-hydroxycholesterol (Fig. 4). Activity increased linearly through 50 min stage 2 incubation time and continued to rise more slowly through 120 min.

Effect of Liposome Size and Phospholipid Composition

When liposomes are prepared as described, particle size is estimated to range from 250 to 300 nM (22), and particles are considered to be small, unilamellar vesicles. Freeze-thaw treatment of these particles is reported to result in an increase in size to 1400 ± 350 nM (23). Both small and freeze-thaw-treated liposomes (0.5 mg/assay asolectin; 500 μM cholesterol) were prepared and used for the assay of 7α-OHase activity. Little effect on 7α-OHase activity in response to this size difference (31.7 ± 2.4 pmoles/min mg for control vs. 29.1 ± 1.0 pmoles/min mg for freeze-thaw) was observed.

Effect of phospholipid composition of liposomes was examined using various purified phospholipids to prepare liposomes (Table 3). In all cases 7α-OHase activity was significantly lower than the activity of asolectin-solubilized substrate, indicating that changes in liposome charge to neutral and increasingly negative liposomes were not paramount in the presentation of cholesterol to the microsomal protein.

DISCUSSION

Use of asolectin to prepare liposome-solubilized substrate for assay of 7α-OHase provides a method for routine assay of the enzyme which avoids several common associated problems. An earlier report of the use of cholesterol-lecithin liposomes in measuring the 7α-OHase activity of acetone extracted microsomal preparations demonstrated the potential of this method but failed to explore ideal conditions for liposome

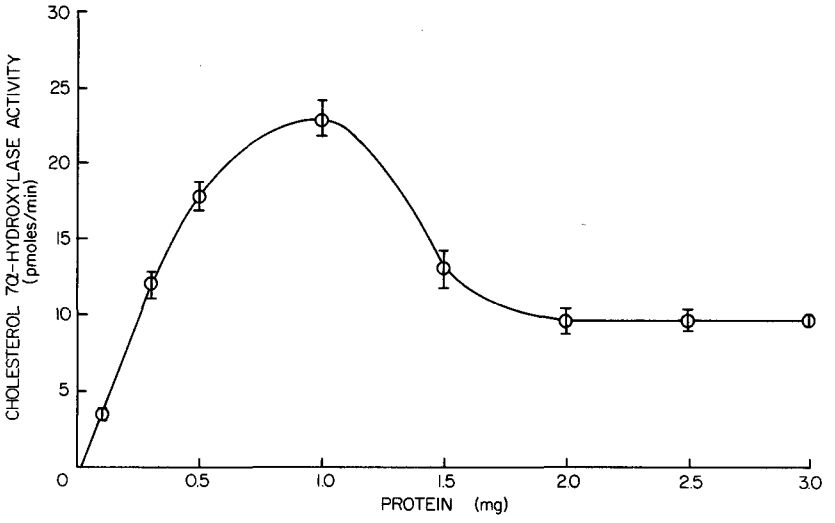

FIG. 3. Effect of increasing amounts of microsomal protein on activity of cholesterol 7α-hydroxylase. Cholesterol (180 μM) solubilized with 0.5 mg/assay asolectin and incubated (stage 1) for 60 min at 37 C. Stage 2 incubation time of 30 min at 37 C.

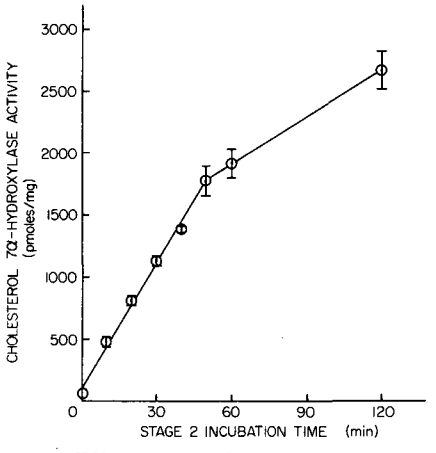

FIG. 4. Effect of stage 2 incubation time on cholesterol 7α-hydroxylase activity. Cholesterol (180 μM) solubilized with 0.5 mg/assay asolectin and incubated (stage 1) with microsomal suspension for 60 min at 37 C.

TABLE 3

Effects of Phospholipid Composition of Liposomes on Cholesterol 7α-Hydroxylase Activity

Phospholipid[a]	Specific activity of 7α-OHase (pmoles/min mg)
Asolectin	45.3 ± 3.5[b]
PC:PS (1:3, v/v)	25.8 ± 0.5
PC:PS (1:1, v/v)	28.9 ± 3.5
PC:PS (3:1, v/v)	31.1 ± 5.8
PC:PE (1:1, v/v)	30.1 ± 1.7
PE:PS (1:3, v/v)	34.4 ± 7.0
PE:PS (1:1, v/v)	36.3 ± 1.4
PE:PS (3:1, v/v)	30.7 ± 1.4

[a]Cholesterol (500 μM) combined with phospholipid listed (0.5 mg/assay) to form liposomes. Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine.

[b]Mean ± standard deviation, n = 3 for all except asolectin, in which n = 7.

preparation or other incubation conditions (15). The assay reported here employs a stage 1 incubation of microsomal protein with substrate (60 min) allowing equilibration of exogenous and endogenous pools of cholesterol to help provide a substrate pool of constant specific activity. Asolectin appears to be the best choice of phospholipid for liposome solubilization of substrate, with optimum 7α-OHase activity occurring when 0.5 mg asolectin/assay was employed. A 30 min stage 2 is then initiated by adding a NADPH generating system, sufficient time to make the assay mechanically practical while not reducing enzyme activity (Fig. 4). For routine assay, a substrate concentration of 180 μM is recommended, since this level results in near maximal activity and is beyond a concentration for which enzyme activity would be significantly altered by small changes in added cholesterol. This minimizes the cost of the assay while maintaining its reliability.

Use of liposomes for substrate solubilization reduces the variability among replicates. Coefficient of variation for replicates (usually three)

of assays employing TW (1.5 mg TW/assay; 180 μM cholesterol) averaged 44.9% and ranged from 11.8 to 74.1% (n = 8) while for those using liposomes under similar conditions (0.5 mg asolectin/assay; 180 μM cholesterol) the range was 1.2 to 7.6% with a mean of 4.2% (n = 9). This improvement in precision of measurement increases the ability to detect changes induced by more subtle effectors such as diet.

These results also indicate that significant variation in activity exists among various microsomal preparations, i.e., between animals, in spite of the above mentioned reduced variation within microsomal samples. However, if we correct the data for between-microsomal preparation differences by expressing individual observations as a percentage of an observation common to all three preparations, the data are very consistent. For example, Figure 5 contains the data for the effects of substrate concentration expressed as a percentage of activity at a substrate concentration of 300 μM. Expressed in this way the data for the three preparations become almost superimposed, indicating that in spite of variation in observed activity among microsomal preparations, the relative activity of the enzyme in response to substrate concentrations seems to be constant. Large variation between animals has been reported for a related enzyme, hydroxy-methylglutaryl-coenzyme A reductase (24). This variability can cause difficulty in detecting subtle differences in enzyme activity, necessitating use of methodology which minimizes within sample variation.

In summary, these results suggest that use of phospholipid liposomes for substrate solubilization in the assay of 7α-OHase allows preincubation with enzyme effectors without the enzyme inhibition previously observed with TW while providing better precision. These improvements are achieved while avoiding use of equipment often not available for routine assays and methods which employ harsh treatment of microsomes to remove endogenous cholesterol. However, this method does not account for differences in endogenous pool sizes among microsomal preparations and must be used carefully when such changes are expected. Removing the inhibitory effects of TW in a method which can be employed routinely in most laboratories outweighs the possible negative effects of differences in endogenous cholesterol concentration, especially in view of the potential damage to the enzyme or its regulatory mechanisms caused by extraction procedures.

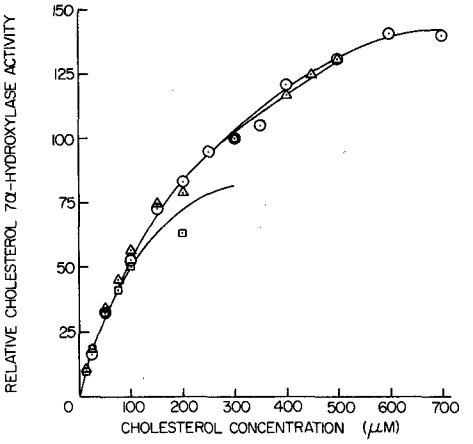

FIG. 5. Effect of substrate concentration on relative cholesterol 7α-hydroxylase activity. Conditions as in Fig. 2 with activity expressed as a percentage of activity at 300 μM substrate concentration for each microsomal preparation.

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COMMUNICATIONS

Norflurazon—An Inhibitor of Essential Fatty Acid Desaturation in Isolated Liver Cells

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ABSTRACT

Norflurazon is a herbicide known to inhibit carotene biosynthesis and linolenic acid biosynthesis in plants. In the present work, the effect of norflurazon on the metabolism of essential fatty acids was studied in isolated rat liver cells and in rat liver microsomes, incubated with [1-^{14}C] labeled linolenic acid (18:3, n-3), dihomogammalinolenic acid (20:3,n-6) and eicosapentaenoic acid (20:5, n-3). Norflurazon (0.1 mM, 1.0 mM) was found to inhibit essential fatty acid desaturation. The Δ6 desaturation is inhibited more efficiently than the Δ5 and Δ4 desaturation. The chain elongation of essential C_{18} fatty acids to their C_{20} and C_{22} homologs was not inhibited by norflurazon.
Lipids 20:719-722, 1985.

INTRODUCTION

Norflurazon (4-chloro-5-methylamino-2-[3-trifluoromethylphenyl]-pyridazin-3[2H] one, SAN 9789) is a commonly used herbicide known to inhibit the carotene biosynthesis (1). Several workers have shown that in plants, norflurazon also inhibits the desaturation of linoleic acid (18:2,n-6) to linolenic acid (18:3,n-3), without affecting the rate of desaturation of oleic acid or stearic acid (2-4).

In animals, dietary linoleic acid and linolenic acid are converted to longer and more desaturated fatty acids, mainly to arachidonic acid (20:4,n-6) and docosahexaenoic acid (22:6,n-3), respectively. The aim of this work was to investigate whether norflurazon inhibits animal fatty acid desaturation.

MATERIALS AND METHODS

Norflurazon (Fig. 1) was from Sandoz A.G., Basel, Switzerland. Labeled fatty acid substrates were purchased from the Radiochemical Center, Amersham, U.K. ([1-^{14}C]linolenic acid) and from New England Nuclear, Boston, Massachusetts ([1-^{14}C]dihomogammalinolenic acid and [1-^{14}C]eicosapentaenoic acid). The specific activity of labeled fatty acid was 7 mCi/mmol. Parenchymal liver cells and liver microsomes were prepared from male weanling rats of the Wistar strain (from Møllegård Laboratory, Denmark). The animals were fed a semisynthetic diet deficient in essential fatty acids (5) with 15 wt% hydrogenated coconut oil for more than six weeks. Isolated liver cells were prepared according to the method of Seglen (6). The concentration of the cells was approximately 5×10^6 cells/ml, and 90-95% were viable, as measured by resistance to uptake of tryphan blue. Cells were incubated in an oxygenated suspension medium (7) with 1.5% (w/v) bovine serum albumin and 10 mM (+)-lactate (from Sigma Chemical Co., St. Louis, Missouri). One ml of the cell suspension (in a total volume of 2 ml) was incubated with 200 nmole of labeled fatty acid. When indicated, the hepatocytes were preincubated for 20 min with norflurazon (dissolved in methanol) or methanol. The concentration of norflurazon was 0.01 mM, 0.1 mM or 1.0 mM, with final concentrations of methanol of 0.1%, 0.2% or 2%, respectively. Microsomes were separated according to the method of Marcell et al. (8) by centrifugation at 105,000 × g for 2 hr. Each labeled substrate fatty acid, bound to bovine serum albumin, was incubated in a concentration of 100 nmoles per ml. Each incubation in 1 ml of a 15 mM Hepes-1 mM EGTA-0.25 M sucrose solution contained in μmoles: ATP, 10; CoA, 0.3; NADH, 1.0; magnesium chloride, 10; phosphate buffer (pH 7.4), 15, and microsomes (5 mg of protein). When indicated, norflurazon (1.0 mM) or methanol was added.

The measurements of radioactive CO_2 and

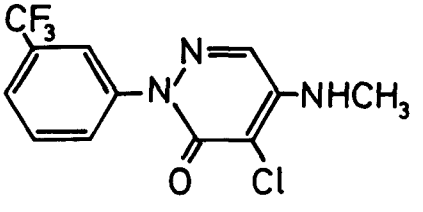

FIG. 1. The chemical structure of Norflurazon.

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TABLE 1

Effect of Norflurazon on the Pattern of ^{14}C-Labeled Fatty Acids in Hepatocytes Incubated with [^{14}C]Linolenic Acid

	Additions				
		Norflurazon dissolved in methanol			Control with methanol (2.0%)
	No addition	0.01 mM	0.1 mM	1.0 mM	
Fatty acid in phospholipid + triacylglycerol fraction					
18:3	68.4 ± 6.1	67.2 ± 6.5	85.4 ± 7.1	154.0 ± 0.4	68.7 ± 5.7
18:4	4.9 ± 0.5	4.0 ± 0.7	3.8 ± 0.3	4.2 ± 0.1	9.2 ± 1.1
20:3	1.5 ± 0.5	1.2 ± 0.3	1.5 ± 0.1	2.5 ± 0.2	2.1 ± 0.1
20:4	8.1 ± 0.5	8.2 ± 0.1	8.7 ± 0.3	6.8 ± 0.8	11.1 ± 1.0
20:5	63.1 ± 4.1	67.8 ± 7.0	53.4 ± 5.3	11.7 ± 0.3	64.5 ± 3.2
22:5	9.7 ± 0.3	9.0 ± 0.3	9.6 ± 1.2	6.4 ± 0.2	10.0 ± 1.0
22:6	22.7 ± 0.9	24.1 ± 1.9	21.8 ± 0.3	3.8 ± 0.3	23.6 ± 1.3
Δ6-Desaturase activity	61.6	62.8	53.6	18.7	63.8
Δ5-Desaturase activity	95.1	92.5	86.9	76.3	90.0
Δ4-Desaturate activity	70.1	72.8	69.4	37.3	70.0
Chain elongation of C_{18} to C_{20}	58.9	60.7	51.6	16.1	58.5
Chain elongation of C_{20} to C_{22}	33.9	32.8	37.0	47.0	34.3
Oxidation products	22.1 ± 1.1	19.0 ± 1.2	16.0 ± 0.8	8.7 ± 0.2	23.3 ± 2.3
Phospholipids	82.4 ± 0.6	82.0 ± 0.2	79.1 ± 0.4	51.1 ± 0.8	75.9 ± 0.5
Triacylglycerols	93.5 ± 2.1	96.9 ± 0.7	103.0 ± 0.9	138.4 ± 0.6	96.5 ± 2.2
Free fatty acids	2.1 ± 0.4	2.1 ± 0.2	1.9 ± 0.1	1.7 ± 0.4	2.5 ± 1.0

The incubation conditions were as described in Materials and Methods. Labeled linolenic acid (0.2 mM) and hepatocytes (24.1-24.4 mg of protein) were incubated for 60 min. The results are given as nmol of ^{14}C-labeled fatty acid esterified, oxidized or remaining as free linolenic acid substrate.

Means ± S.D. of two parallel analyses from three different livers are given. Activities of desaturases and chain elongations are given as per cent.

radioactive acid soluble products were performed as described by Christiansen (9). The lipids were extracted by the method of Folch et al. (10) and separated on silicic acid thin layer plates (Stahl H+) (hexane/diethylether/glacial acetic acid, 80:20:1, v/v/v). Aliquots of the total lipid extracts were transmethylated (11) and analyzed by radio gas chromatography (12). Cellular protein was determined according to the method of Lowry et al. (13).

RESULTS AND DISCUSSION

Δ6 Desaturating activity was studied by using [1-^{14}C]linolenic acid (18:3,n-3) as substrate for isolated liver cells from rats fed an essential fatty acid deficient diet. The main metabolites recovered in triacylglycerol and phospholipids were eicosapentaenoic acid (20:5,n-3) and docosahexaenoic acid (22:6,n-3) (Table 1). Nearly all the fatty acid substrate was metabolized, either esterified or oxidized under the conditions used.

With norflurazon (0.1 mM, 1.0 mM) present in the medium, the total amount of desaturated and chain-elongated fatty acids formed decreased markedly compared with the control. Table 1 shows that the activity of Δ6 desaturase, calculated as the sum of 18:4(n-3), 20:4 (n-3), 20:5(n-3), 22:5(n-3) and 22:6(n-3) in per cent of the sum of these five fatty acids and 18:3(n-3), decreased by 70% in the presence of norflurazon (1.0 mM). The fatty acids most markedly reduced were 20:5(n-3) and 22:6(n-3), with a concomitant increase of the amount of unaltered linolenic acid esterified.

Isolated microsomes also were used to study the effect of norflurazon. Table 2 shows that the Δ6 desaturating activity, calculated as 18:4 (n-3) formed from 18:3(n-3), decreased by 67% in the presence of norflurazon (1.0 mM). The solvent alone (methanol) had no effect on Δ6 desaturation.

The present experiments show that norflurazon inhibits desaturation of essential fatty acids in liver cells and in microsomes under the conditions used. The results cannot differentiate between an effect on the Δ6 desaturase itself or an effect on the electron transfer from NADH via cytochrome b_5 to the desaturase.

Several workers have shown that the regulation of Δ5 desaturase activity differs from the

mechanisms regulating the Δ6 desaturase (12, 14). In order to study the effect of norflurazon on Δ5 desaturation, labeled dihomogammalinolenic acid (20:3,n-6) was used as substrate. Table 3 shows that norflurazon (1 mM) decreased the conversion of 20:3(n-6) to arachidonic acid (20:4,n-6) modestly (by 15%). In experiments with 18:3(n-3) as substrate, where the Δ5 desaturation was calculated as the sum of 20:5(n-3), 22:5(n-3) and 22:6(n-3) in per cent of these three and 20:4(n-3) (Table 1), norflurazon decreased the Δ5 desaturating activity, but also in this case less efficiently than observed with Δ6 desaturation.

TABLE 2

The Effect of Norflurazon on the Desaturation of [^{14}C]Linolenic Acid in Rat Liver Microsomes

Addition	μmoles of 18:4/min/mg protein
No addition	21.2 ± 0.5
Norflurazon (1 mM)	7.1 ± 0.5
Methanol	24.2 ± 0.9

The incubation conditions were as described in Materials and Methods. The results are expressed as μmole [^{14}C]18:4 formed per min per mg protein.

Means ± S.D. of three parallel analyses from two different livers are given.

It has been shown previously that the Δ4 desaturase, which converts 22:5(n-3) to 22:6(n-3) and, less efficiently, 22:4(n-6) to 22:5(n-6) is similar to the Δ6 desaturase, in that essential fatty acid deficiency increases the activity of both enzymes (12). This is not the case with Δ5 desaturase (12,14). Δ4 desaturase activity was studied by using eicosapentaenoic acid 20:5(n-3) as substrate which is chain-elongated to 22:5 (n-3) and subsequently desaturated to 22:6(n-3). Table 3 shows that norflurazon (1 mM) caused a moderate reduction of the desaturation of 22:5(n-3) to 22:6(n-3). With 20:3(n-6) as the substrate, norflurazon was found to inhibit the desaturation of 22:4(n-6) to 22:5(n-6) to a similar extent. Only small amounts of 22:4(n-6) and 22:5(n-6) were formed in these experiments.

Norflurazon did not inhibit the chain elongation reactions. The elongation of C_{20} to C_{22} fatty acids even seemed to be stimulated in the presence of norflurazon. Thus with 20:5(n-3) as the substrate the sum of 22:5(n-3) and 22:6 (n-3) (Table 3) increased with norflurazon. Only a minor fraction of the fatty acids used was oxidized in the present experiments. The oxidation of 18:3(n-3) was reduced by norflurazon. The oxidation of 20:5(n-3) was reduced slightly, and the oxidation of 20:3(n-6) seemed to be unaffected by the inhibitor.

TABLE 3

The Effect of Norflurazon on the Pattern of ^{14}C-Labeled Fatty Acids in Hepatocytes Incubated with [^{14}C]Dihomogammalinolenic Acid or [^{14}C]Eicosapentaenoic Acid

	Fatty acid substrate				
	20:3(n-6)			20:5(n-3)	
	No addition	Norflurazon (1.0 mM)		No addition	Norflurazon (1.0 mM)
Fatty acid in phospholipid + triacylglycerol fraction					
20:3	40.9 ± 1.5	53.1 ± 0.6	20:5	114.3 ± 0.5	100.1 ± 1.7
20:4	107.7 ± 0.4	91.0 ± 0.3	22:5	27.5 ± 0.6	39.5 ± 2.5
22:4	2.0 ± 0.1	4.8 ± 0.2	22:6	24.2 ± 0.6	27.6 ± 2.1
22:5	1.4 ± 1.0	2.5 ± 0.2			
Δ5-Desaturase activity	73.0	64.9			
Δ4-Desaturate activity	41.0	34.2		47.0	40.0
Chain elongation of C_{20} to C_{22}	3.0	7.4		31.1	40.1
Oxidation products	48.2 ± 1.5	48.5 ± 0.3		34.3 ± 0.5	30.1 ± 0.2
Phospholipids	121.5 ± 0.5	88.4 ± 0.6		109.1 ± 0.3	76.5 ± 0.2
Triacylglycerols	29.7 ± 0.5	61.7 ± 0.8		59.8 ± 0.2	91.5 ± 0.2
Free fatty acids	1.4 ± 0.1	1.3 ± 0.1		1.7 ± 0.1	1.8 ± 0.1

The incubation conditions were as described in Materials and Methods. Labeled dihomogammalinolenic acid or eicosapentaenoic acid (0.1 mM) and hepatocytes (24.1-24.4 mg of protein were incubated for 60 min.

The results are given as nmol of ^{14}C-labeled fatty acid esterified, oxidized or remaining as free fatty acid substrate.

Means ± S.D. of two parallel analyses from three different livers are given. Activities of desaturases and chain elongations are given as per cent.

Tables 1 and 3 show that parallel to the inhibition of desaturases, norflurazon increased the incorporation of labeled fatty acids in triacylglycerol and reduced the incorporation in phospholipids. This change in distribution of labeled fatty acids between phospholipids and triacylglycerol probably is a consequence of the effects of norflurazon on the desaturation reactions. Thus, the intact 18:3(n-3) substrate is mainly esterified in triacylglycerol, while quantitatively dominating products formed after Δ6 desaturation, 20:5(n-3) and 22:6(n-e), are preferentially esterified in the phospholipids (12). Intact 20:3(n-6) is mainly esterified in triacylglycerol, while arachidonic acid formed by Δ5 desaturation is very efficiently directed to the phospholipids (12). With 20:5(n-3) as substrate, norflurazon stimulates the chain elongation to 22:5(n-3), which is preferentially esterified in triacylglycerol (14). The present experiments show that norflurazon inhibits the desaturation of essential fatty acids in liver cells and in liver microsomes under the conditions used. Δ6 desaturase is inhibited more efficiently than the other two desaturases studied.

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Free, Esterified and Glucosidic Sterols in Cocoa Butter

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ABSTRACT

Sterol lipids of cocoa butter (cocoa beans *Lome Tongo*) were fractionated into free sterols, steryl esters (SE), steryl glucosides and acylated steryl glucosides (ASG). 4-Desmethyl, 4-methyl and 4,4′-dimethyl sterols or triterpene alcohols, which were isolated as free sterols or which resulted from hydrolysis, were determined by thin layer chromatography-flame ionization detection and identified by gas chromatography and combined gas chromatography-mass spectroscopy. Free sterols comprise the main sterol fraction in cocoa butter. Esterified sterols amount to 11.5% of total sterols and glucosidic sterols to 16.3%. Fatty acids and D-glucose from hydrolysis of esters and glucosides were analyzed. The fatty acids of SE and ASG are richer in unsaturated fatty acids than cocoa butter total fatty acids.
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INTRODUCTION

Previous studies on the sterols in cocoa butter dealt only with analysis of 4-desmethyl, 4-methyl and 4,4′-dimethyl sterols or triterpene alcohols from unsaponifiable matter (1-4). Sterol lipids in vegetable fats exist as free sterols, steryl esters (SE), steryl glucosides (SG) and acylated steryl glucosides (ASG). The composition of sterol lipids has been investigated in few vegetable fats (5-7), although such information is significant in the study of sterol distribution in plants and plant products.

In this investigation, the four forms of sterol lipids in cocoa butter were fractionated. The 4-desmethyl, 4-methyl and 4,4′-dimethyl sterols or triterpene alcohols, which were isolated as free sterols or which resulted from hydrolysis of SE, SG and ASG, were determined and identified or tentatively identified. Fatty acids and sugars from hydrolysis of esters and glucosides also were analyzed and their fatty acid composition compared to that of cocoa butter total fatty acids.

EXPERIMENTAL

Extraction and Fractionation

Cocoa butter was extracted from powdered cocoa beans *(Lome Tongo)* (free of shells and seed buds) in a Soxhlet apparatus with acetone for 24 hr and fractionated by column chromatography into SE, free sterols and glucosides. Each column contained 50 g Kieselgel 60 (70-230 mesh) (Merck), activated for 12 hr at 110 C, and on top a layer of dry Na_2SO_4. Ten g of cocoa butter in petroleum ether were added to each column and eluted (2 ml/min) with (a) 500 ml petroleum ether. (b) 700 ml 1%, (c) 700 ml 2%, (d) 700 ml 5%, and (e) 700 ml 20% (v/v) Et_2O in petroleum ether, (f) 700 ml $CHCl_3$ (washed with distilled H_2O and dried over Na_2SO_4), and (g) 1200 ml acetone. Solvents were removed with a rotatory evaporator, and fractions were dried with benzene, dissolved in $CHCl_3$ and analyzed by silica gel thin layer chromatography (TLC) using petroleum ether/Et_2O/acetic acid (80:20:1, v/v/v) as developing solvent. Plates were sprayed with 5% v/v H_2SO_4 in EtOH and visualized by charring at 140 C for 3-5 min. R_f values of individual components were compared with those of the following standards: cholesterol (Merck), cholesteryl palmitate (Aldrich), and steryl glucoside (Supelco). Fractions (a) and (b) contained SE and triglycerides, fractions (c), (d) and (e) free sterols, fraction (f) free sterols and glucosides, and fraction (g) glucosides.

Fractions (a) and (b) were combined and purified by Kieselgel 60 column chromatography. The column was developed with 250 ml petroleum ether (rejected), 200 ml 0.5%, and 100 ml 1% v/v Et_2O in petroleum ether (SE fraction), and 100 ml 1% v/v Et_2O in petroleum ether (rejected).

Fraction (f) dissolved in 10% v/v Et_2O in petroleum ether was refractionated by Kieselgel 60 column chromatography. The column was developed with 250 ml 10%, 250 ml 15%, and 150 ml 20% v/v Et_2O in petroleum ether (free sterols); 150 ml 20% and 150 ml 50% v/v Et_2O in petroleum ether, and 150 ml $CHCl_3$ (rejected), and 450 ml $CHCl_3$ (glucosides).

Glucosides were separated into SG and ASG by preparative TLC using $CHCl_3$/MeOH (95:12, v/v) as developing solvent (8).

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Hydrolysis of SE and ASG Analysis of Methyl Esters

Each of the esterified fractions (SE and ASG) was hydrolyzed with 0.4 N methanolic KOH (5 ml/10 mg of ester) for 6 hr at 37 C. The reaction mixture was cooled and added to $CHCl_3/H_2O$ ($CHCl_3/H_2O$/MeOH, 4:1:2, v/v/v), where the sterol components passed into the $CHCl_3$ phase. The aqueous methanol phase was acidified to pH 2 with 6 N HCl, fatty acids extracted with Et_2O and methylated with 10% methanolic boron trifluoride. Methyl esters were extracted with n-heptane and analyzed by gas chromatography (GC) (Tracor 550: 10% EGSS-X on Gaschrom Q, 1.8 m, 3 mm i d).

Hydrolysis of SG—Analysis of Sugars

Each of the glucosidic fractions was refluxed with 5% methanolic HCl for 6 hr at 100 C, cooled, and the hydrolyzed sterols extracted four times with Et_2O, after addition of H_2O to the reaction mixture. The residual methanol layer was passed through a column of Amberlite IR-4B (Mallinkrodt) to obtain pure methyl glucoside (5) which then was refluxed with 2 N HCl for 2 hr at 100 C.

Qualitative analysis of sugars was performed on Kieselgel G thin layer plates impregnated with boric acid using acetone/n-BuOH/H_2O (5:4:1, v/v/v) as developing solvent (9).

Fractionation, Determination and Analysis of Sterols

Each of the free sterol fractions was separated on silica gel thin layer plates by development with Et_2O/petroleum ether/formic acid (50:50:0.5, v/v/v) and visualized by spraying with 0.1% w/v 2′, 7′-dichlorofluorescein in EtOH. Fractions were separated into 4-desmethyl, 4-methyl and 4,4′dimethyl sterols or triterpene alcohols. Bands were scraped off the plates and eluted with dry Et_2O. Quantitative determination of the separated sterols was performed by thin layer chromatography-flame ionization detection (TLC-FID), Iatroscan TH-10, Iatron Lab., Inc. Each fraction was diluted to 10 ml with $CHCl_3$, and 0.5 ml of the solution was transferred to a test tube, where a suitable amount of the standard solution (cholesteryl palmitate) was added so that the concentration of total sterols and the standard would be approximately the same in the final solution. Most of the solvent was removed under N_2, and a suitable amount of the solution containing ca. 15 μg of sterols was applied on a quartz rod with sintered coating of silica gel (chromarods SII, Iatron Lab., Inc.). The chromarods were developed with petroleum ether/Et_2O/acetic acid (80:20:1, v/v/v), dried and scanned. The calibration factor, 0.83 relative to cholesteryl palmitate, was determined by analyzing known mixtures of cholesterol and cholesteryl palmitate to correct for nonlinearity of instrument response.

Each sterol fraction was analyzed by GC (Sigma 2, Perkin Elmer; glass column packed with 3% OV-17 on Gaschrom Q, 100-120 mesh, 1.8 m, 3 mm i d; oven temp 255 C) and its percent composition determined. Relative retention times (RRT) of sterols were calculated relative to cholesteryl acetate. Acetoxy sterols were prepared by acetylation according to the literature (10) and analyzed by GC (stationary phase OV-17) and combined GC-MS (MAT 44, 0.7 mA, 78 eV; open tubular SE-54 glass column, 25 m, 0.25 mm i d; oven temp 200 C). RRT values of steryl acetates were calculated again relative to cholesteryl acetate (stationary phase OV-17).

Quantitative Estimation of Total SE, SG, and ASG

The weight of each fraction was estimated from the data obtained by TLC-FID by using a mean MW for 4-desmethyl, 4-methyl and 4,4′-dimethyl sterols or triterpene alcohols and for fatty acids and the MW of D-glucose.

RESULTS AND DISCUSSION

Free sterols comprise the main sterol fraction in cocoa butter (Table 1). Esterified sterols amount to 11.5% of total sterols and glucosidic sterols to 16.3%. Literature data show that the quantitative relation among sterol fractions lies in wide ranges (5,6). SE in cocoa butter appear relatively richer in 4-methyl and 4,4′-dimethyl sterols or triterpene alcohols than the free sterols. It was not possible to isolate any 4-methyl sterols or triterpene alcohols from SG or ASG.

The sterol components were identified by their RRT values relative to cholesteryl acetate (stationary phase OV-17), ΔR_{Ac} values (RRT of steryl acetate/RRT of sterol), spectral characteristics and literature data (10-13).

4-Desmethyl Sterols

The 4-desmethyl sterol composition of the four sterol fractions isolated from cocoa butter is shown in Table 2. Six 4-desmethyl sterols were identified or tentatively identified in the free sterol fraction. Five mass spectra (A-E) were obtained which were assigned as follows:

A. MS m/e (rel int): 368{M-AcOH}$^+$ (100),

TABLE 1

Sterol Fractions from 100 g of Cocoa Butter

Fraction		mg of sterols in the fraction	mg of fraction	Total
Free sterols	4-Desmethyl sterols	178.0	178.0	
	4-Methyl sterols	9.9	9.9	207.9
	Triterpene alcohols	20.0	20.0	
SE	4-Desmethyl sterols	23.9	39.0	
	4-Methyl sterols	2.1	3.4	54.0
	Triterpene alcohols	7.1	11.6	
ASG	4-Desmethyl sterols	20.0	40.0	40.0
SE	4-Desmethyl sterols	27.0	37.5	37.5

TABLE 2

4-Desmethyl Sterols in Cocoa Butter

Acetate	RRT[a] OV-17	ΔR_{Ac}[b]	Percentage			
			Free	SE	SG	ASG
Cholesterol	1.00	1.33	1.1	2.7	1.2	1.0
Brassicasterol	1.16	1.34	—	1.4	0.3	0.9
Campesterol	1.32	1.33	12.3	10.7	8.6	8.2
Stigmasterol	1.44	1.32	23.4	12.6	16.9	17.0
Sitosterol Stigmastanol	1.66	1.33	60.3	67.2	70.7[c]	70.5[c]
Isofucosterol (or fucosterol)	1.84	1.32	2.9	4.6	2.3	2.4
Δ^7-Stigmastenol	1.96	1.34	—	0.8	tr	tr

[a]Relative to cholesteryl acetate.

[b]RRT of steryl acetate/RRT of sterol.

[c]Sitosteryl acetate only.

353(20), 275 (5), 260(36), 255{M-AcOH-side chain}$^+$ (20), 247(33), 228(5), 213(17) to cholesteryl acetate (RRT 1.00).

B. MS m/e (rel int): 382 {M-AcOH}$^+$ (100), 367(20), 289 (1), 274(23), 261(22), 255{M-AcOH-side chain}$^+$ (18), 247(21), 228(5), 213(16) to campesteryl acetate (RRT 1.32).

C. MS m/e (rel int): 394 {M-AcOH}$^+$ (100), 379(8), 351(10), 296(4), 281(11), 255{M-AcOH-side chain}$^+$ (88), 228(9), 213(23), 211(16) to stigmasteryl acetate (RRT 1.44).

D. MS m/e (rel int): 396{M-AcOH}$^+$ (100), 381(17), 303(1), 288(25), 275(21), 255(29), 228(8), 213(27) to sitosteryl acetate (RRT 1.66).

E. Belonged also mainly to sitosteryl acetate, but the co-occurrence of the ions at m/e 398{M-AcOH}$^+$, 383{M-AcOH-CH_3}$^+$, 305{M-AcOH − part of A+B ring}$^+$, 215 {M-AcOH-side chain-D ring}$^+$ (base peak) suggested possibly the presence of stigmastanyl acetate (RRT 1.69), and the ions at m/e 394{M-AcOH}$^+$, 296{M-AcOH-part of side chain }$^+$ (base peak), 253{M-AcOH-side chain-2H}$^+$, 211{M-AcOH-side chain-2H-D ring}$^+$ suggested the presence of isofucosterol or fucosterol (RRT 1.84).

The above MS data agree with literature values (11-13).

Gas chromatographic analysis of the 4-desmethyl sterols (as acetates) from hydrolysis of SE, SG and ASG also showed the presence of brassicasteryl acetate (RRT 1.16) and Δ^7-stigmastenyl acetate (RRT 1.96), both reported previously in the literature (2). We were not able to obtain their mass spectra because of insufficient material.

Five mass spectra also were obtained with the 4-desmethyl sterols from hydrolysis (SE, SG and ASG), similar to the spectra of the free

4-desmethyl sterols. However, spectrum E from the SG and ASG 4-desmethyl sterols did not show the presence of the ions assigned tentatively to stigmastanyl acetate.

Differences between the free and the esterified 4-desmethyl sterol fraction concern mainly the presence of brassicasterol and Δ^7-stigmastenol and the higher percentages of cholesterol and isofucosterol (or fucosterol) in the esterified fraction. Also, the ratio of sitosterol/stigmasterol is 2.5 in the free and 5.5 in the esterified 4-desmethyl sterols. Similar differences were reported in the literature for other lipids of vegetable origin (6,7,14).

The 4-desmethyl sterol composition is similar in SG and ASG, probably because of their biogenetic relation. The glucosidic sterols are richer in sitosterol than the free 4-desmethyl sterols.

4-Methyl Sterols

The free and esterified 4-methyl sterol composition of cocoa butter is shown in Table 3. Ten 4-methyl sterols were identified or tentatively identified in the free sterol fraction. The following 10 mass spectra (A-J) were obtained:

A. MS m/e (rel int): 456{M}$^+$ (26), 411{M-CH_3}$^+$ (100), 381{M-AcOH-CH_3}$^+$ (73), 287 {M-side chain-D ring-CH_2}$^+$ (15), 283{M-AcOH-side chain}$^+$ (11), 241 (11), 227(17) was assigned to 31-norlanost-8-en3β-yl acetate (RRT 1.11).

B. The second spectrum resulted from a mixture of two 4-methyl steryl acetates with RRT 1.33. The main component, MS m/e (rel int): 442{M}$^+$ (90), 427(15), 382{M-AcOH}$^+$ (10), 367(15), 269 {M-AcOH-side chain}$^+$ (100), 242(18), 227(39), was identified as lophenyl acetate. The presence of the ions at m/e 454{M}$^+$, 439{M-CH_3}$^+$, 379{M-CH_3-AcOH}$^+$, 301{M-side chain-D ring}$^+$, and 287{M-side chain-D ring − CH_2}$^+$ on the spectrum also indicated the occurrence of 31-norlanosteryl acetate.

C. MS m/e (rel int): 456(12), 427(24), 411(18), 396(90), 381(100), 341(24), 288(48), 283(54) remained unidentified.

D. The fourth spectrum resulted from a mixture of two 4-methyl steryl acetates. The main component, MS m/e (rel int): 468{M}$^+$ (48), 453{M-CH_3}$^+$ (100), 393(53), 287{M-side chain-CH_2-D ring}$^+$ (34), 241(28), 227(34), was identified as obtusifoliyl acetate (RRT 1.48). The ions also at m/e 394{M-AcOH}$^+$, 379{M-AcOH-CH_3}$^+$, 286 (characteristic of 9,19 cyclopropane ring), and 283{M-AcOH-side chain}$^+$ indicated the presence of 31-norcycloartenyl acetate (RRT 1.57).

E. MS m/e (rel int): 454{M}$^+$(6), 439(6), 379 (6), 370{M-part of side chain}$^+$ (30), 327{M-side chain-2H}$^+$ (100), 269(33), 267(15), 227{M − AcOH-side chain-D ring}$^+$ (21) was identified as gramisteryl acetate (RRT 1.77).

F. The sixth spectrum resulted from a mixture of two 4-methyl steryl acetates. The main component was identified as cycloeucalenyl acetate, the spectrum of which also was obtained independently (spectrum G). The presence of the ions at m/e 456{M}$^+$, 441{M-CH_3}$^+$, 381 {M-AcOH-CH_3}$^+$, 302{M-side chain-part of D ring}$^+$, 287{M-side chain-D ring}$^+$, 269{M-AcOH-side chain}$^+$, 242{M-AcOH-side chain-part of D ring}$^+$, 227 {M-AcOH-side chain-D

TABLE 3

4-Methyl Sterols in Cocoa Butter

Acetate	RRT[a] OV-17	ΔR_{Ac}[b]	Percentage Free	Percentage SE
31-Norlanost-8en-3β-ol	1.11	1.30	9.7	4.5
Lophenol 31-Norlanosterol	1.33	1.30	13.0	15.3[c]
Obtusifoliol	1.48	1.29	15.1	16.9
31-Norcycloartenol	1.57	1.31	6.0	14.0
Methyllophenol Cycloeucalenol Gramisterol	1.77	1.30	17.4	17.7
Ethyllophenol	2.15	1.30	8.4	3.8
Citrostadienol	2.40	1.30	30.4	27.8

[a]Relative to cholesteryl acetate.

[b]RRT of steryl acetate/RRT of sterol.

[c]Lophenyl acetate only.

ring}$^+$, suggested the occurrence of methyllophenyl acetate (RRT 1.77).

G. MS m/e (rel int): 468{M}$^+$ (8), 453(12), 408(100), 393(77), 300 (characteristic of 9,19 cyclopropane ring) (28), 281(30), 241{M-AcOH-side chain-D ring}$^+$ (30), 227(40) was identified as cycloeucalenyl acetate (RRT 1.77).

H. MS m/e (rel int): 470{M}$^+$(90), 455(18), 410(12), 395(15), 329(45), 269{M-AcOH-side chain}$^+$ (100), 242(33), 227(41) was assigned to ethyllophenyl acetate (RRT 2.15).

I. MS m/e (rel int): 468{M}$^+$ (3), 453(6), 393(8), 370{M − part of side chain}$^+$ (61), 327{M-side chain-2H}$^+$(100), 269(10), 267(20), 242(11), 227(20) was identified as citrostadienyl acetate (RRT 2.40).

J. MS m/e (rel int): 456(40), 396(50), 381(45), 302(35), 269(100), 227(65) remained unidentified.

The above MS data agree with literature values (11-13).

The eight mass spectra which were obtained with the 4-methyl sterols of the esterified fraction were similar to those we obtained with the free 4-methyl sterols except that they did not show the presence of 31-norlanosterol and of the two unidentified 4-methyl sterols.

Comparison of the 4-methyl sterols isolated from the free and esterified sterol fraction shows that the ratio of 31-norlanostenol/31-norcycloartenol is higher in free 4-methyl sterols (1.6) than in their esters (0.3). It is to be noted that these two 4-methyl sterols are formed by different biosynthetic pathways.

4,4′-Dimethyl Sterols or Triterpene Alcohols

The 4,4′-dimethyl sterols or triterpene alcohols isolated from the free and esterified sterol fractions of cocoa butter are shown in Table 4. Five triterpene alcohols were identified in each case, and one remained unidentified. It was not possible to obtain the mass spectrum of the unidentified triterpene alcohol with RRT 2.22 and ΔR_{AC} 1.20, because of insufficient material.

The following five mass spectra (A-E) were obtained:

A. Ms m/e (rel int): 470{M}$^+$ (15), 455(52), 410(3), 395(100), 357{M-side chain}$^+$ (4), 330(3), 315(10), 301(6), 297(15), 270(10), 255(21), 241(31) assigned to lanostenyl acetate (RRT 1.31).

B. MS m/e (rel int): 468{M}$^+$, 453,408,218(100), 203(41) assigned to β-amyrin acetate (RRT 1.66).

C. MS m/e (rel int): 470{M}$^+$ (15), 455(30), 410(100), 395(95), 357{M-side chain}$^+$ (10), 297(95), 288(95) identified as cycloartanyl acetate (RRT 1.56).

D. MS m/e (rel int): 468{M}$^+$ (20), 453(15), 408(100), 393(78), 365(49), 357{M-side chain}$^+$ (8), 355(18), 315(5), 301(12), 297{M-AcOH-side chain}$^+$ (29), 295(22), 286 (characteristic of 9, 19 cyclopropane ring) (91), 255(22), 241(24) identified as cycloartenyl acetate (RRT 1.88).

E. MS m/e (rel int): 482{M}$^+$(10), 467(8), 439(6), 422(100), 407(70), 379(44), 357{M-side chain}$^+$ (8), 315(12), 301(43), 300 (characteristic of 9,19 cyclopropane ring) (90), 297(44), 295(14), 270(11), 255(13) identified as 24-methylenecycloartanyl acetate (RRT 2.09).

The above MS data agree with literature values (11-13).

No appreciable differences were observed between the two triterpene alcohol fractions.

Fatty Acids

The fatty acid composition of SE and ASG is similar, but differs from that of the cocoa butter total fatty acids (Table 5). Percentages of the unsaturated fatty acids, i.e. linoleic and linolenic acid, are higher (5-fold) in SE and ASG. Similar results on fatty acid composition were reported in the literature for other seeds, but were not evaluated (5-7).

TABLE 4

4,4′-Dimethyl Sterols or Triterpene Alcohols in Cocoa Butter

Acetate	RRT[a] OV-17	ΔR_{Ac}[b]	Percentage Free	Percentage SE
Lanostenol	1.31	1.20	0.5	1.0
Cycloartanol	1.56	1.22	7.6	8.0
β-Amyrin	1.66	1.19	1.8	6.2
Cycloartenol	1.88	1.23	60.6	64.1
24-Methylene-cycloartanol	2.09	1.22	23.5	19.7
Unidentified	2.22	1.20	6.0	1.0

[a]Relative to cholesteryl acetate.

[b]RRT of steryl acetate/RRT of sterol.

TABLE 5

Fatty Acids in Cocoa Butter

Methylester of	Cocoa butter %	SE %	ASG %
Palmitic acid	27.6	23.8	20.3
Palmitoleic acid	0.7	1.1	2.0
Stearic acid	32.9	23.9	25.7
Oleic acid	35.0	29.2	32.3
Linoleic acid	2.8	17.5	15.3
Linolenic acid	0.8	3.6	3.6
Arachidic acid	0.2	0.9	0.8

Sugars

D-glucose was found to be the only sugar in glucosidic sterols. This is similar to literature data for other vegetable seed oils (15).

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Induced Chemiluminescence of Oxidized Fatty Acids and Oils

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ABSTRACT

The injection of a strong organic base into milligram quantities of fats and oils dissolved in methylene chloride results in a burst of chemiluminescence whose peak intensity is a function of the previous thermal oxidation history and of the degree of unsaturation of the starting material. The flash of this induced chemiluminescence can be 10^8 times higher than the steady-state "spontaneous" chemiluminescence. The kinetics of the induced chemiluminescence are first order in concentration and second order in time. The emission spectrum is broad and extends into the near infrared. A model based on dioxetane chemiluminescence is proposed to explain the observed kinetics.
Lipids 20:729-734, 1985.

INTRODUCTION

The oxidation of fatty acids and oils by molecular oxygen results in products which affect the palatability as well as the toxicity of the original material. The accumulation of these products in the fatty acids or oils gives rise to a "spontaneous," weak chemiluminescence (CL) which has been used as an assay for the deterioration of the fats and oils (1-4). It is possible, by reacting these same partially oxidized fats and oils with strong organic base, to increase the initial intensities of the observed CL by a factor of 10^8 over the intensities of the "spontaneous" CL, providing a more sensitive assay for the deterioration products. In this paper, we describe the conditions for producing this induced CL, its concentration and temporal kinetics, the dependence of the CL upon the duration of thermal oxidation and the degree of unsaturation of the fats and oils and the emission spectra of the CL. We propose a possible mechanism for the production of the CL.

MATERIALS AND METHODS

Commercially available refined soybean oil with no additives was used in all cases. Analytical grade methyl esters of the fatty acids oleic, linoleic and linolenic were from Sigma Chemical Co., St. Louis, Missouri; methylene chloride, reagent grade from J.T. Baker Chemical Co., Philipsburg, New Jersey; K tert-butoxide from Aldrich Chemical Co., Milwaukee, Wisconsin; tert-butanol from Matheson, Coleman & Bell, Norwood, Ohio.

Preparation of Heated Oils

Thermal oxidation of soybean oil, 140-180 C, was accomplished by continuous stirring, 50 rpm, on a hot plate in 125 ml Ehrlenmeyer flasks in the presence of air. Aliquots were withdrawn at various times and assayed for chemiluminescence. Test tubes 15 x 100 mm containing 2 ml of methyl ester of fatty acid were immersed in a constant temperature oil bath at 180 C and sampled as a function of time. Because only small quantities of fatty acid methyl esters were available, these samples were not stirred. Therefore, the thermal oxidation was not maximized in these cases. Anaerobic heating of oils was accomplished in sealed ampules. Peroxide values were measured as described in Official and Tentative Methods of the American Oil Chemists' Society, Cd 8-53, μmole (O_2)/g oil.

Chemiluminescence Induction

Measurements were made in triplicate. CL was induced by injection of 100 μl of K tert-butoxide (0.1 M) dissolved in tert-butanol with a constant rate spring-loaded syringe, CR-700-200, Hamilton Co., Reno, Nevada, into 2 ml of methylene chloride at room temperature, containing 50 μl of previously heated oil delivered with a positive displacement microdispenser, Drummond Scientific Co., Broomall, Pennsylvania.

Chemiluminescence Detection

CL was measured in several different photometer geometries. In most cases, kinetics of CL emission were followed in a light box with a single phototube, RCA type 1P21 or equivalent. A double photometer for red and blue emission kinetics used two phototubes viewing the source at 180°. A Gencom EMI 9781 and a Hamumatsu R1477P-HA in combination with a Corning CS 5-58 bandpass filter and a Hoya R-60 glass cut-on filter were used to isolate "blue" and "red" portions of the CL spectrum, respectively (5-8). In all cases, an Analog Devices model 42K electrometer amplifier in combina-

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tion with a Brush 220 recorder, Gould Inc., Cleveland, Ohio, provided the fast time response required (frequency response 100 Hz) to record the mixing times. A previously calibrated, sealed, ^{14}C-activated phosphor was used to calibrate the photometers to units of photons per second equivalent to a broad blue emission peaking at 490 nm (5,6).

Low intensities of CL were determined in a liquid scintillation counter light collection geometry using an RCA 4501 phototube and an Ortec photon counting system, calibrated for photon emission against the quantum yield of the Luminol CL reaction (9).

Chemiluminescence Emission Spectrum

An interference filter spectrometer (10) employing two cavity, 10 nm bandpass interference filters from 380-800 nm in combination with a Gencom EMI 9863A (S-20 response) phototube was used to determine the CL emission spectrum. CL reactions were induced in (15 x 45 mm) shell vials with the optically clear bottom of the vial positioned 26 mm above the upper surface of the interference filters. The filters were in close proximity to the photocathode of the vertically mounted end window photomultiplier. Triplicate (2.05 ml) reaction mixtures in the shell vials containing 50 µl of previously heated soybean oil in methylene chloride were induced by injection of 50 µl of K tert-butoxide (0.1 M).

Total CL was integrated over 20 seconds. The dark noise (20 C) during this interval was 1228 counts ± 4% (C.V.). The signal-to-noise ratio at the wavelength of maximum spectral intensity was approximately 12.

RESULTS

The presence of molecular oxygen during heating is a specific requirement for the observed CL. Soybean oil and methyl ester fatty acids subjected to identical heating regimes in the absence of air gave no measurable CL over unheated fresh samples. However, the K tert-butoxide induced CL itself does not require molecular oxygen. Previously heated samples of soybean oil reacted with K tert-butoxide under air-bubbled and argon-bubbled conditions gave the same intensities of CL.

The peak CL as a function of amount (µmoles) of K tert-butoxide added to the reaction mixture containing 50 µl of soybean oil is shown in Figure 1A. Under conditions of excess K tert-butoxide, successive dilution of the soybean oil in methylene chloride resulted in a linear dependence of peak CL upon the oil concentration as shown in Figure 1B.

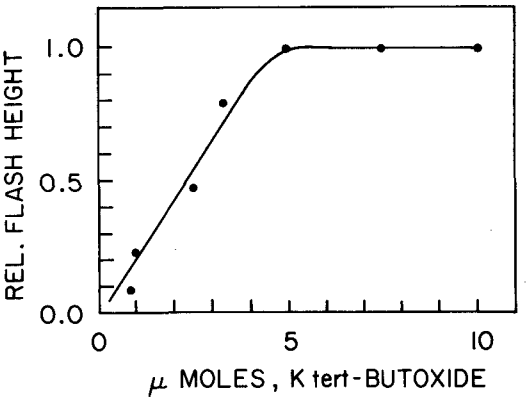

FIG. 1A. Relative CL flash height at room temperature as a function of K tert-butoxide added to a reaction mixture, final volume 2.150 ml, containing 2 ml methylene chloride and 50 µl previously heated (10 hr, 180 C) soybean oil.

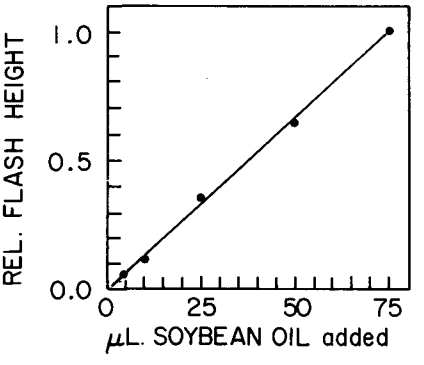

FIG. 1B. Relative CL flash height as a function of total amount of added soybean oil. Reaction mixture and conditions same as Figure 1A except 100 µl K tert-butoxide (0.1 M) used to induce all samples.

The relative spectral distribution of the induced CL of previously heated soybean oil is shown in Figure 2A. This spectrum is extremely broad, extending from 380 nm to 800 nm with a half width of 260 nm. It would be unlikely for a single species of product molecule to produce such a broad emission spectrum. Therefore, we measured coincidentally the kinetics of the blue and red regions of the spectrum, using the double filter-photometer technique (5-8). As shown in Figure 2B, the time for the initial rise of the CL to a peak value is the same for both the blue and red regions. However, the intensity of the blue CL appears to decay initially at a significantly higher rate. After 2s, both blue and red emissions decay at essentially the same rates.

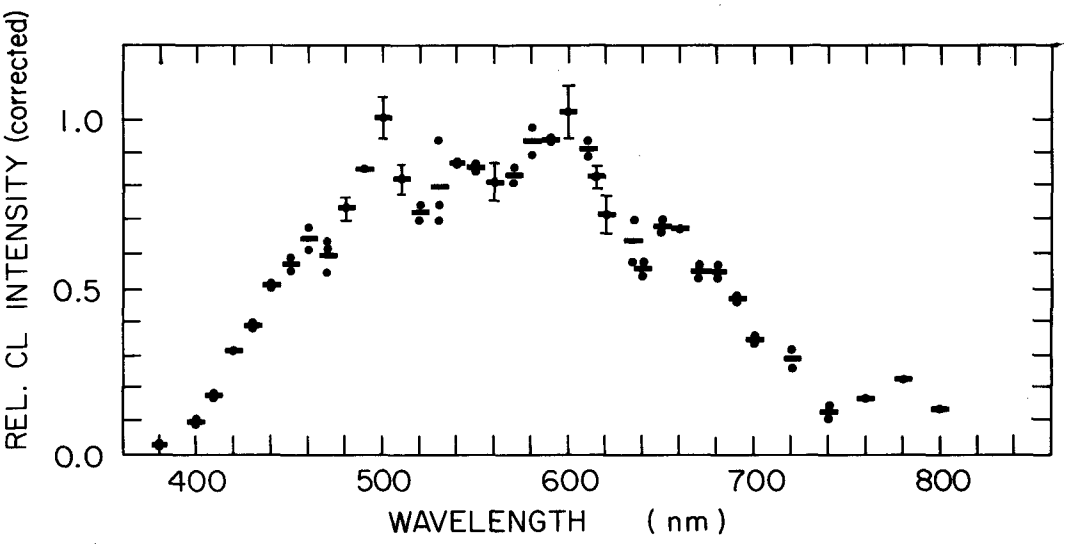

FIG. 2A. Corrected CL spectral emission of previously heated soybean oil using 10 nm full width at half maximum intensity (FWHM) bandpass interference filters. Individual determinations (n) are plotted for each filter except where n > 3, in which case, error bars represent standard error of the mean (± SEM). Horizontal bars positioned vertically at the mean represent the bandwidth of each filter.

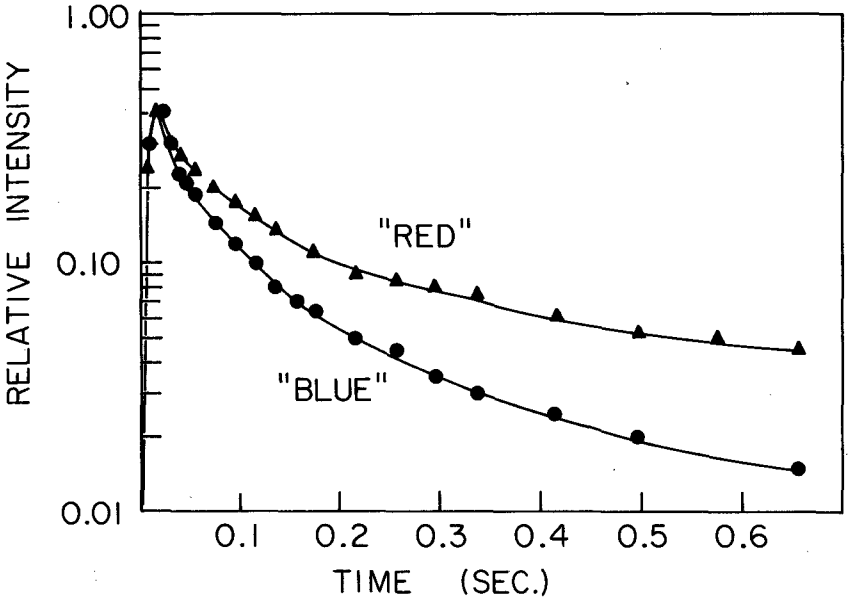

FIG. 2B. Kinetics of K tert-butoxide induced CL of soybean oil using a "RED" Hoya R-60 sharp cut on ($T_{600\ nm}$ = 50%) filter and a "BLUE" Corning Cs-5-58 (405 nm peak, 60 nm FWHM) bandpass glass filter to isolate opposite ends of the emission spectrum.

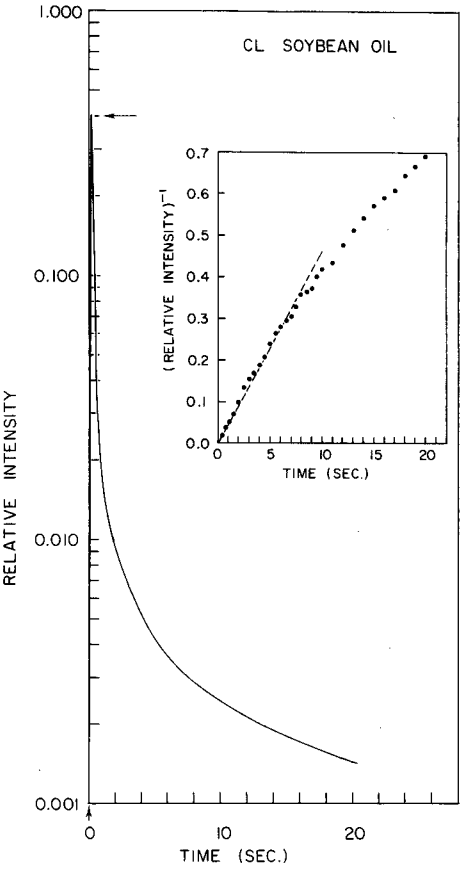

FIG. 3. Typical relative intensity of a K tert-butoxide induced CL reaction of previously heated soybean oil reacted at room temperature. Arrows indicate time of injection (t=0) and a very rapid peak flash height. Inset, circles (•) represent the same CL data plotted as the reciprocal of flash intensity as a function of time. The dashed line represents an ideal second order decay.

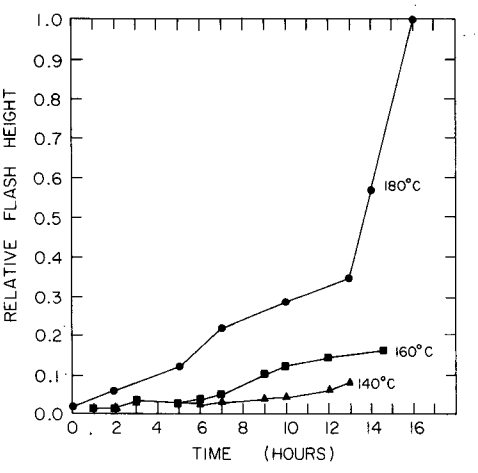

FIG. 4. Relative CL flash heights of soybean oil continuously heated at three different temperatures for varying times up to 16 hr. Aliquots were removed periodically and induced to CL with K tert-butoxide.

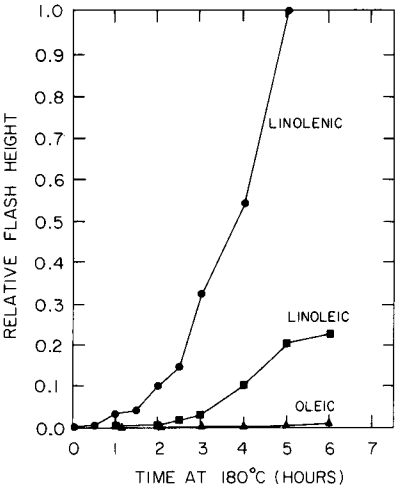

FIG. 5. Relative CL flash heights of oleic, linoleic and linolenic methyl esters continuously heated for up to 6 hr at 180 C. Aliquots were removed periodically and induced to CL with K tert-butoxide.

The time course of K tert-butoxide induced CL of previously heated soybean oil is shown in Figure 3. There is a peak intensity of CL at ca. 20-40 ms, reproducible but dependent upon the rate of mixing of the injected K tert-butoxide. In the inset, Figure 3, the data are plotted to compare the measured kinetics with that of a second order reaction (dashed line). Despite the rapid decay, the intensity of the induced CL, even after one hr, is still 15× higher than that of the "spontaneous" CL (absence of K tert-butoxide).

The effects upon the induced CL of soybean oil under prolonged heating at different temperatures and under aerated (stirring) conditions are shown in Figure 4. The effects of the degree of unsaturation upon the induced CL, and inferentially, upon the yield of oxidized products, are shown in Figure 5, for methyl oleate (18:1), methyl linolate (18:2) and methyl linolenate (18:3), heated at 180 C.

Finally, the comparisons between the CL Flash Heights and the peroxide values obtained according to AOCS Cd 8-53, for soybean oil heated at 60 C and at 180 C, are shown in Figures 6A and 6B. As can be seen, the peroxide value assay is not applicable at the higher temperature.

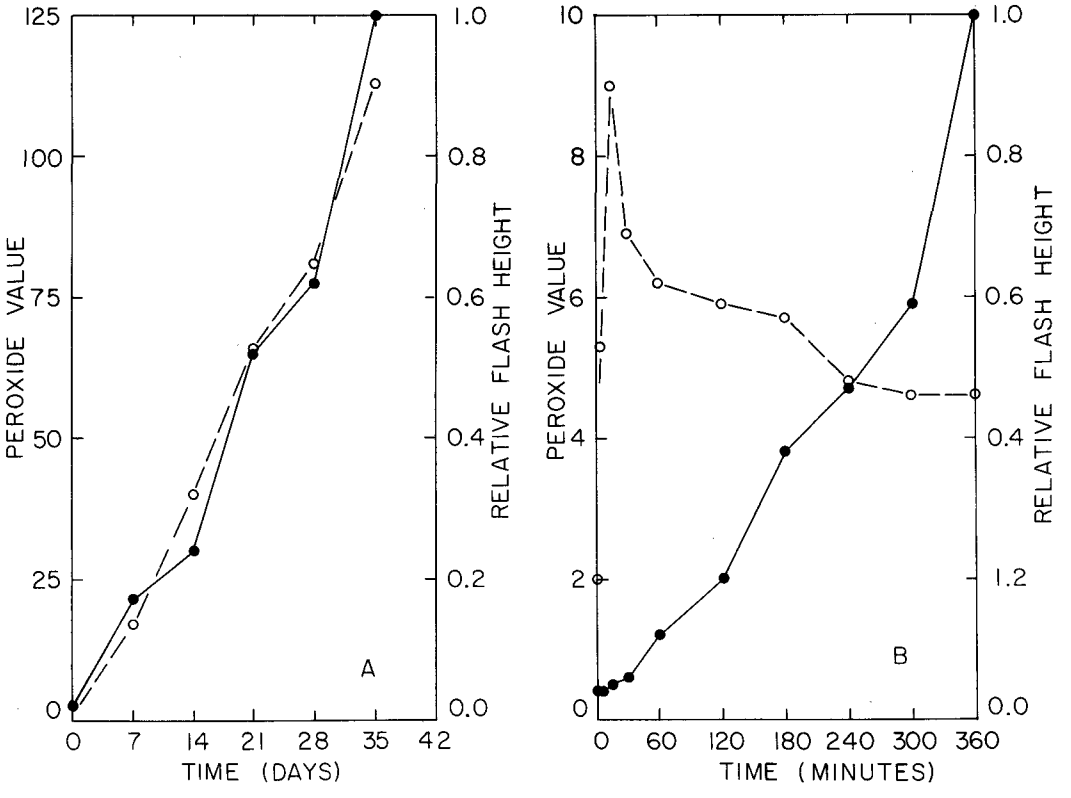

FIG. 6. Relative CL flash height at room temperature (•) and peroxide value (μmole O_2/g oil) (○) versus time of heating of aliquots of soybean oil. A. 60 C; B. 180 C.

DISCUSSION

From the kinetic plots in Figure 1B and the inset to Figure 3, the intensity of CL is first order in concentration but second order in time. We assume that in the presence of strong organic base, thermal oxidation products are cyclized to dioxetanes which are chemiluminescent when their decomposition is spontaneous (intramolecular electron exchange CL). Luminescence does not occur when dioxetane decomposition results from a bimolecular electron exchange. These relationships take the forms:

$$CL = k_1 \text{ (Dioxetane)} \quad [1]$$

and

$$-\frac{d}{dt}\text{(Dioxetane)} = k_1 \text{ (Dioxetane)} + k_2 \text{ (Dioxetane)}^2 \quad [2]$$

where Eq. [1] represents a first order concentration dependence and Eq. [2] implies the second order temporal dependence. The assumption that thermal oxidation products can be cyclized to dioxetanes does not exclude other chemiluminescent intermediates. Equations [1] and [2] therefore represent models for CL that fit the observed kinetics.

From Figure 3 and the absolute calibration of our photon detectors, soybean oil, heated at 180 C for 10 hr, produced a peak of induced CL of approximately 5×10^7 photons s^{-1} per mg of oil. The absence of a requirement for molecular oxygen in the induced CL suggests that the function of the strong organic base may be to catalyze the cyclization of peroxides to dioxetanes, which are known chemiluminescent intermediates (11). The emission of a significant amount of red light from oxidation products of fats and oils with relatively low conjugation does not appear to have a simple explanation. Possibly polymerized product molecules could possess sufficient electronic orbital overlap to lower the energies of the excited states sufficiently to result in this red region spectral emission. The kinetics of decay of the CL of the red and blue regions shown in Figure 2B might be explained by assuming that

there exists one rate constant for the emission of both a red-emitting component and a blue-emitting component, responsible for the constant red/blue intensity ratio for $t > 2$ s, and a second, shorter lived, blue-emitting component, with negligible intensity at $t > 2$ s. The high sensitivity for detection of induced CL may have application to the assay of lipid peroxidation in cells and tissues. This technique is at present being compared with published analytical methods for measuring oxidation in fats and oils.

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Cholesterol Transport and Uptake in Miniature Swine Fed Vegetable and Animal Fats and Proteins. 1. Plasma Lipoproteins and LDL Clearance[1]

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ABSTRACT

In a 2 × 2 factorial arrangement, miniature pigs were fed four diets containing vegetable protein/fat (soybean) and animal protein (egg white)/fat (beef tallow) to demonstrate the effects of protein and fat source on total plasma cholesterol, lipoprotein distribution, low density lipoprotein (LDL) composition, and plasma clearance of LDL-cholesterol and protein. Beef tallow consumption resulted in greater plasma cholesterol concentration, decreased LDL-cholesterol concentration, and a lower LDL-cholesterol to LDL-protein ratio than did consumption of soybean oil. High density lipoprotein (HDL)-cholesterol concentration was increased by beef tallow consumption. Cholesterol percentage by weight in LDL was significantly greater in pigs consuming soybean oil than those consuming beef tallow. Percentages by weight of protein, triglyceride and phospholipid in LDL were not significantly different in any group. Dietary protein source had no significant effect on total plasma cholesterol concentration, lipoprotein concentration or LDL composition. Egg white consumption decreased fractional catabolic rate and irreversible loss of LDL-cholesterol and LDL-protein when compared with consumption of soy protein. Dietary fat source had no consistent effect on LDL clearance from plasma. Dietary fat and protein seemed to influence lipoprotein metabolism by different mechanisms. Fat source altered lipoprotein concentration and LDL composition, whereas protein source affected the removal rate of LDL from plasma.
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INTRODUCTION

Vegetable products, especially soybean oil and soy protein, have been found hypocholesterolemic by many researchers when compared with animal products consumed by many species (1-4). Dietary composition also influences LDL composition and clearance of LDL from plasma (5-9). However, many of these studies used purified or semipurified diets, whereas fats and proteins usually are consumed as part of a mixed diet. Interactions between fat and protein sources in a mixed diet, as well as interactions with other dietary constituents, may play an important role in determining the overall effect of diet composition. Our research group has shown that feeding pigs complex diets containing soy protein and soybean oil resulted in no significant change in total plasma cholesterol while decreasing LDL-cholesterol concentrations and increasing HDL-cholesterol concentrations, compared with diets containing beef protein and beef tallow (10). These diets were not purified, and contained fiber, carbohydrates and other components naturally present in feedstuffs.

Our interest was the influence of dietary fat and protein sources in the context of diets composed of ingredients that might be consumed by people living in the U.S. Our objectives were to determine, in miniature pigs, the effects of source of dietary protein and fat: (i) on cholesterol concentration in plasma and its distribution between the major plasma lipoproteins; (ii) on LDL composition, and (iii) on clearance of LDL-cholesterol and LDL-protein from the plasma. Swine were chosen because they are naturally omnivorous and are similar to humans in cardiovascular and digestive physiology and blood and lipid profile (11-13).

MATERIALS AND METHODS

Animals

Sixteen Hormel miniature pigs (Sinclair Research Farm, St. Louis, Missouri) and eight NIH strain (originally Yucatan × Hormel) miniature pigs (Bisland Memorial Swine Breeding Farm, Iowa State University, Ames, Iowa) were divided into four groups balanced for breed and sex (16 males, 8 females). Pigs were maintained at 22 C and housed individually in stainless steel cages with mesh floors, suspended over sawdust.

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Diets

Beginning at six wk of age, pigs were fed four isocaloric, isonitrogenous diets (six pigs/diet) in a 2 × 2 factorial arrangement. Diets contained raw egg white (Crystal Whip egg whites, Crystal Food Inc., Minneapolis, Minnesota) or soy protein isolate (Supro 620, Ralston Purina Co., St. Louis, Missouri) as the main protein source and rendered beef tallow (Country Meat Market, Granger, Iowa) or soybean oil (Edsoy, Staley Mfg. Co., Decatur, Illinois) as the main fat source. Egg white was selected as the animal protein because it is readily available, contains virtually no lipid, and is a sizable component of human diets in the U.S. The ingredient and nutrient compositions of the diets are shown in Tables 1 and 2. Diets were supplemented with lysine, methionine, vitamins and minerals to meet the 1979 National Research Council recommendations for growing pigs consuming high-energy diets (14). Additional biotin (Hoffmann-La Roche Inc., Nutley, New Jersey) was added to the diets containing egg white to overwhelm the biotin-binding capacity of the avidin in the raw egg white. In addition, dried egg yolk (standard dried egg yolk solids, Oskaloosa Food Production Corp., Oskaloosa, Iowa) was added as necessary to provide each pig with approximately 30 mg of cholesterol daily per kg of body weight. Diets were fed once daily for six wk. Pigs were weighed weekly, and feeding rates were adjusted weekly so that each pig's daily dry matter allowance was 3.5% of its body weight. Water was available ad libitum.

Plasma Lipids

Each week, pigs were fasted for 12 hr and blood samples were collected by way of orbital sinus puncture. Disodium ethylene diamine tetraacetate was used as an anticoagulant. Plasma was recovered by centrifugation and stored at approximately 2 C. Lipoproteins were separated from plasma by density adjustment and ultracentrifugation at 18 C (Model L-2 or L8-70 ultracentrifuge, Ti 50.3 rotor, Beckman Instruments Inc., Irvine, California) by the method of Havel et al. (15). Total cholesterol and lipoprotein cholesterol concentrations were determined enzymatically (Centrifichem Cholesterol Reagent-6 min, Baker Instruments Corp., Pleasantville, New York). Protein concentrations of LDL were determined by the method of Lowry as modified by McDonald and Chen (16), using bovine serum albumin as a standard. Initial (wk 0) samples of LDL and those collected at wk 3 and 6 also were analyzed enzymatically for triglyceride concentrations (Tri-

TABLE 1

Ingredient Composition of Diets

Ingredient	Diet[a] EW/SO	EW/BT	SP/SO	SP/BT
	% of dry matter			
Ground shelled corn	55.0	55.0	58.5	58.8
Raw egg white	19.2	19.7	—	—
Soy protein isolate	—	—	15.5	15.7
Soybean oil	17.0	—	117.0	—
Beef tallow	—	17.0	—	17.0
Dried egg yolk	2.9	2.4	2.9	2.4
L-Lysine-HCl	0.6	0.7	0.4	0.4
DL-Methionine	—	—	0.4	0.4
Vitamin/mineral supplements[b]	5.2[c]	5.3[c]	5.2	5.3
	% of calories			
Corn	47.1	47.1	50.1	50.3
Egg white	15.7	16.1	—	—
Soy protein isolate	—	—	12.7	12.9
Soybean oil	32.5	—	32.5	—
Beef tallow	—	33.0	—	33.0
Egg yolk	4.7	3.8	4.7	3.8

[a]EW = egg white, SP = soy protein, SO = soybean oil, BT = beef tallow.

[b]Supplements were added so that diets contained at least these concentrations of the following nutrients, expressed as percent or amount per kg diet (dry matter basis): Ca, 0.72%; P, 0.61%; Na, 0.11%; Cl, 0.14%; K, 0.29%; Mg, 0.04%; Fe, 89 mg; Zn, 89 mg; Mn, 3.3 mg; Cu, 5.5 mg; I, 0.16 mg; Se, 0.17 mg; vitamin A, 1944 IU; vitamin D, 222 IU; vitamin E, 12 IU; vitamin K, 2.2 mg; riboflavin, 3.3 mg; niacin, 20 mg; pantothenic acid, 12 mg; choline, 1000 mg; vitamin B_{12}, 17 μg.

[c]Additional biotin was added at 96 μg biotin/100 g egg white dry matter.

TABLE 2

Nutrient Composition of Diets

Component	Diet[a] EW/SO	EW/BT	SP/SO	SP/BT
Dry matter (DM), %	40.3	39.8	92.3	92.3
Digestible energy, kcal/100 g DM	463.0	463.0	463.0	463.0
Protein, % DM	21.0	21.1	21.0	21.0
Fat, % DM	21.4	21.0	21.7	21.3
Fiber, % DM	1.4	1.4	1.5	1.5
Ash, % DM	5.6	5.9	5.7	5.9
Cholesterol, mg/ 100 g DM	87.0	87.0	87.0	87.0
P/S[b]	2.3	0.2	2.3	0.2

[a]See Table 1.

[b]Proportion by weight of polyunsaturated to saturated fatty acids in diets.

glycerides [500 nm], Fisher Scientific Co., Fair Lawn, New Jersey) and colorimetrically for inorganic phosphorus concentrations (Phosphorus Rapid Stat® Diagnostic Kit, Lancer Div. of Sherwood Medical, St. Louis, Missouri). An average molecular weight of 800 was used to calculate phospholipid concentrations from inorganic phosphorus.

LDL Labeling

After four wk, additional LDL was isolated from each pig, and the apoprotein component was labeled with [U-^{14}C]sucrose (555 mCi/mmol, Amersham, Arlington Heights, Illinois) by using the technique of Pittman et al. (17,18). This method produces LDL with the radioactively-labeled sucrose covalently bound to the apolipoprotein. Additional plasma was used to label the unesterified cholesterol pool in LDL with [1-α, 2-α (n)-^{3}H]cholesterol (47 Ci/mmol, Amersham, Arlington Heights, Illinois) by using the technique of Schwartz et al. (19). Labeling of LDL was verified by ultracentrifugation techniques (15) and polyacrylamide gel electrophoresis (20).

LDL Kinetics

The [^{3}H]cholesterol- and [^{14}C]sucrose-LDL from each pig were combined, and autologous injections (average 1.9 μCi ^{3}H and 1.3 μCi ^{14}C/kg body weight) into the jugular vein were performed after the pigs had consumed the diets for six wk. Serial blood samples were collected for 48 hr from femoral vein catheters inserted surgically at least 5 days before. The specific radioactivities of [^{3}H]cholesterol and [^{14}C]-sucrose in plasma were determined by using standard dual label scintillation counting methods and were fitted to a two-pool model for disappearance by using the NNLIN program of the Statistical Analysis System (SAS) to calculate the half-lives of the curve components, fractional catabolic rate, and irreversible loss for LDL-cholesterol and LDL-protein.

Statistics

Data were subjected to analysis of variance according to the factorial arrangement in a randomized block design by using the SAS procedure GLM to determine the effects of fat and protein source and to test for interactions between fat and protein. Data are expressed as group means with pooled standard errors of the mean.

RESULTS

All pigs readily consumed the feed offered, and all but one grew well and were in good health throughout the experiment. One pig in the soy protein/beef tallow group developed pneumonia; no data from this pig are included in statistical analyses.

The mean weekly total plasma cholesterol concentrations are shown in Figure 1. Beef tallow caused a significant increase ($P < 0.05$) in total plasma cholesterol by wk 3, which continued until the end of the experiment. There was no difference in total plasma cholesterol attributable to type of dietary protein. Diet composition had no significant effect on very low density lipoprotein (VLDL)-cholesterol concentration (data not shown). The effect of the diets on LDL-cholesterol concentration is shown in Figure 2. From wk 4 on, beef tallow consumption resulted in a significant decrease ($P < 0.05$) in the proportion of total cholesterol carried in the LDL fraction. HDL-cholesterol concentration (data not shown) was significantly increased ($P < 0.05$) as a result of beef tallow consumption at wk 5 and 6. The ratio of HDL-cholesterol to LDL-cholesterol concentration (Fig. 3) was significantly greater ($P < 0.05$) at wk 4 and tended to be greater ($P < 0.08$) at wk 5 and 6 when beef tallow was consumed. Protein source had no consistent effect on plasma cholesterol concentration or on VLDL-, LDL- or HDL-cholesterol concentrations.

Concentration of LDL-protein as a function of diet and time is shown in Figure 4. There were no significant effects of either dietary fat

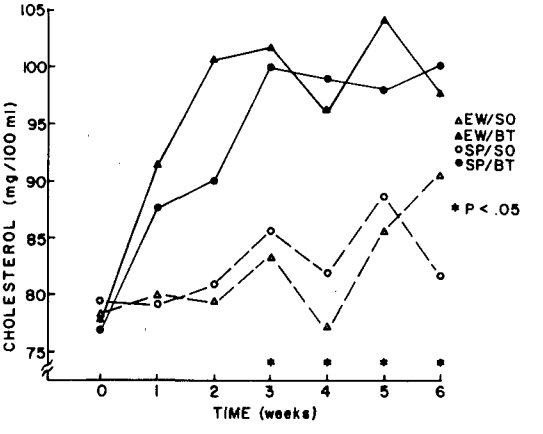

FIG. 1. Mean total plasma cholesterol concentrations. EW = egg white, SP = soy protein, BT = beef tallow, SO = soybean oil, * = fat effect. Each group had six pigs except SP/BT, which had five.

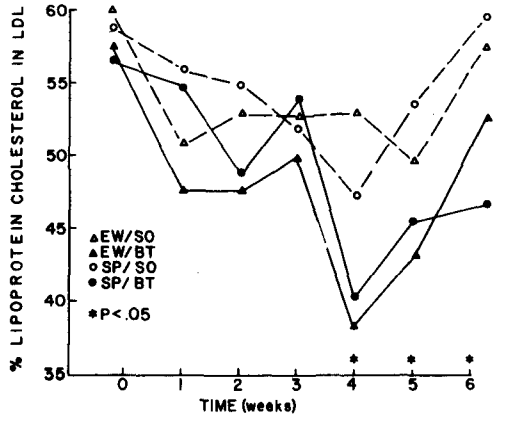

FIG. 2. Mean LDL-cholesterol concentrations, expressed as percent of total lipoprotein cholesterol. EW = egg white, SP = soy protein, BT = beef tallow, SO = soybean oil, * = fat effect. Each group had six pigs except SP/BT, which had five.

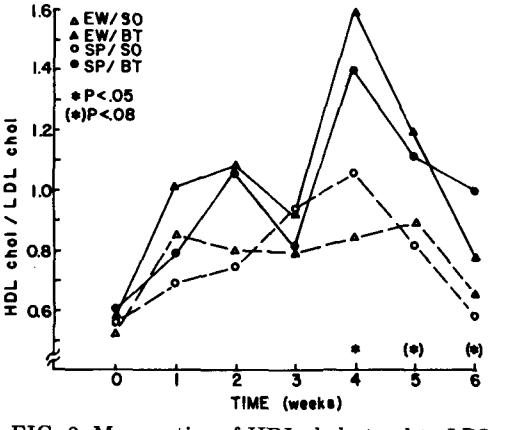

FIG. 3. Mean ratios of HDL-cholesterol to LDL-cholesterol. EW = egg white, SP = soy protein, BT = beef tallow, SO = soybean oil, * = fat effect. Each group had six pigs except SP/BT, which had five.

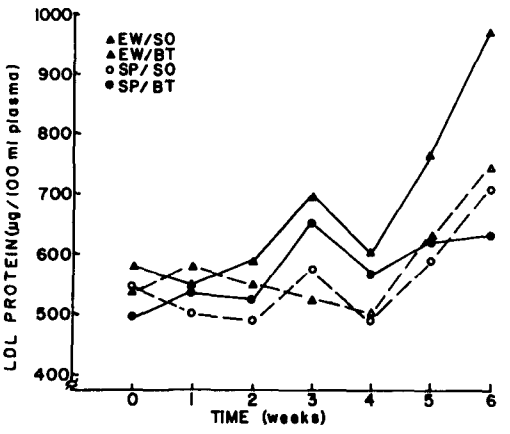

FIG. 4. Mean LDL-protein concentrations. EW = egg white, SP = soy protein, BT = beef tallow, SO = soybean oil. Each group had six pigs except SP/BT, which had five. Although a 50% difference existed between the LDL-protein concentrations in the EW/BT and SP/BT groups at wk 6 (970 vs. 633.4), the great variability within the groups resulted in this difference not being statistically significant (SEM = 181.4 for EW/BT and 113.2 for SP/BT; P = .074).

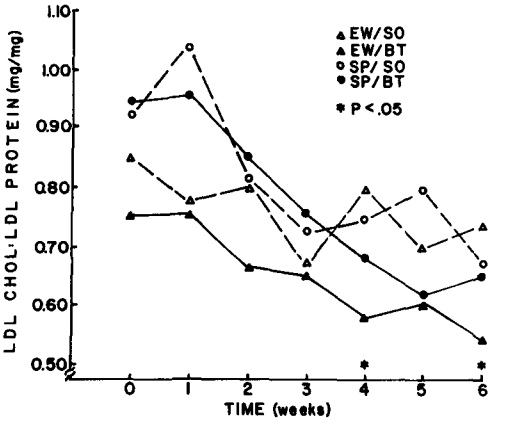

FIG. 5. Mean ratios of LDL-cholesterol to apoprotein. EW = egg white, SP = soy protein, BT = beef tallow, SO = soybean oil, * = fat effect. Each group had six pigs except SP/BT, which had five.

or protein source. The concentration of LDL-protein, however, tended to be greater in both tallow fed groups at wk 3 and 4 and in the egg white/beef tallow group at wk 5 and 6 as well, when compared with both groups consuming soybean oil. The LDL-protein concentration of the soy protein/beef tallow group plateaued during wk 5 and 6, whereas apo-LDL concentrations of the other three groups continued to increase. Figure 5 illustrates the ratio of LDL-cholesterol to LDL-protein. This ratio tended to decline throughout the experiment for all dietary groups. Beef tallow consumption, compared with soybean oil, resulted in a significantly smaller ($P < 0.05$) ratio of cholesterol to protein at wk 4 and 6.

LDL composition is summarized in Table 3. Protein source did not significantly influence LDL composition. Cholesterol, as a percentage of lipoprotein weight, was significantly greater ($P < 0.01$) at the end of the experiment (wk 6) in pigs consuming soybean oil than in pigs consuming beef tallow. Protein percentage increased from wk 0 to wk 6; pigs consuming soybean oil tended to have a smaller percentage ($P < 0.10$) of LDL protein at wk 3, but this difference had disappeared by wk 6. The propor-

TABLE 3

LDL Composition

Wk/LDL component	Diet[a] EW/SO	EW/BT	SP/SO	SP/BT	Pooled SEM
	% weight				
Wk 0					
Cholesterol[b]	30.5	28.7	29.8	28.9	1.4
Protein	26.7	28.5	26.0	25.0	3.3
Triglyceride	7.5	5.9	6.1	5.2	1.0
Phospholipid	35.3	37.0	38.1	41.0	2.9
Wk 3					
Cholesterol	30.2	26.6	27.8	29.6	2.2
Protein	30.0	34.5	29.8	34.1	2.6
Triglyceride	4.5	5.9	4.4	5.2	1.1
Phospholipid	35.3	33.1	38.0	31.2	2.1
Wk 6					
Cholesterol	27.2[c]	21.0	26.9[c]	25.1	1.9
Protein	32.9	35.3	31.6	32.5	2.7
Triglyceride	5.9[d]	10.2[d]	8.2[d]	6.6[d]	1.6
Phospholipid	34.1	33.5	33.3	35.9	2.4

[a]See Table 1.

[b]Free + esterified.

[c]Fat effect, $P < 0.01$.

[d]Fat × protein interaction, $P < 0.05$.

tion of triglyceride tended to be greater ($P < 0.07$) initially in pigs consuming soybean oil, but by the end of the experiment, no consistent effect of dietary fat could be seen. There was, however, a significant interaction ($P < 0.05$) between fat and protein at wk 6. Pigs consuming the egg white/beef tallow diet had the greatest proportion of triglyceride, and those consuming the soy protein/soybean oil diet had the next greatest proportion. Pigs consuming the other two diets had similar triglyceride percentages, with those consuming the soy protein/beef tallow diet being slightly greater. There were no significant dietary effects on phospholipid percentage, which remained constant throughout the study.

Kinetic parameters of LDL-cholesterol and LDL-protein are presented in Table 4. Some difficulty was experienced with maintaining the patency of the femoral vein catheters, and six pigs per diet were not always available for LDL kinetics measurement. The number of pigs consuming each diet for which complete blood samples were collected is included in Table 4. There was no diet effect on the half-life of the first component of the disappearance for either the apoprotein or the cholesterol constituent of LDL. Half-life of the second curve component

TABLE 4

Kinetics of LDL Components

Variable	Diet[a] EW/SO	EW/BT	SP/SO	SP/BT	Pooled SEM
n	5	5	5	3	
LDL-protein					
Half-life, 1st component (h)	0.87	1.13	1.19	1.51	0.35
Half-life, 2nd component (h)	18.1[b]	21.5	15.3[b]	27.9	3.5
Fractional catabolic rate (h^{-1})	0.09	0.08	0.14	0.06	0.03
Irreversible loss (mg/h × kg)	0.12	0.10	0.32	0.12	0.04
LDL-cholesterol					
Half-life, 1st component (h)	0.22	0.24	0.23	0.35	0.07
Half-life, 2nd component (h)	26.6	25.8	26.1	18.2	7.0
Fractional catabolic rate (h^{-1})	0.15	0.18	0.27	0.22	0.07
Irreversible loss (mg/h × kg)	7.87[c]	4.42[c]	13.68	11.64	3.26

[a]See Table 1.

[b]Fat effect, $P < 0.10$.

[c]Protein effect, $P = 0.07$.

for LDL-protein was significantly shorter ($P < 0.05$) in pigs consuming soybean oil than in those consuming beef tallow. Fractional catabolic rate of LDL-protein and LDL-cholesterol tended to be less in pigs fed egg white than those fed soy protein. Irreversible loss of LDL-cholesterol also tended to be lower ($P < 0.10$) in pigs consuming egg white.

DISCUSSION

As in most studies, beef tallow was hypercholesterolemic compared with soybean oil. Egg white did not have a hypercholesterolemic effect in swine compared with soy protein. This is not unexpected because Carroll (4) showed that, of a variety of animal proteins, egg white was the least hypercholesterolemic in rabbits. Any hypercholesterolemic effect of egg white also might have been diluted by interaction with protein provided by the ground corn. Evidence for such interactions between beef and vegetable protein and between casein and soy protein have been presented (4,21). The interaction of protein source with other dietary ingredients, such as carbohydrate or fiber, also may have been a factor. O'Brien and Reiser (22) found no significant difference between the serum cholesterol concentration of rats fed diets containing beef and bread and those fed chow. However, in the same study, rats fed purified diets had significantly greater serum cholesterol concentrations.

Lipoprotein-cholesterol concentration was influenced by dietary fat, but not by dietary protein. LDL-cholesterol concentration decreased and HDL-cholesterol concentration increased when beef tallow was consumed, resulting in an increased HDL to LDL ratio. This could be considered antiatherogenic, because such changes are associated with a decrease in the risk of atherosclerosis and coronary heart disease (23). These changes, however, are different from those observed in other studies. Diets high in polyunsaturated fat decreased both LDL- and HDL-cholesterol concentrations in some cases (24,25), whereas in others, LDL-cholesterol was reduced while HDL-cholesterol remained constant (26–28). In one study, rats fed beef tallow had LDL-cholesterol concentrations similar to those of rats fed corn oil (29). Because the response to polyunsaturated fat may vary, other unknown factors also must play a role in determining the concentration of lipoproteins. Interactions between type of fat and cholesterol and between fiber and fat have been shown (30,31). Other factors are likely to be involved, emphasizing the need to evaluate the effects of dietary factors in the context of complex diets as well as in defined diets. The extent of the influence of the dietary protein source on lipoprotein concentration also needs to be studied further. Experiments with rats (32–34), chickens (35), swine (36) and people (37) showed that dietary protein source had no effect on LDL-cholesterol concentration and only a slight, variable effect on HDL-cholesterol concentration. Other studies (38,39) have shown decreases in LDL-cholesterol when subjects were fed vegetable protein in place of animal protein. It seems that the protein source may have less effect than the type of fat on lipoprotein concentrations. Thus, our results indicating no difference in response between egg white and soy protein are not unexpected.

LDL composition also has been shown to be sensitive to diet composition. Generally, when an effect is seen, polyunsaturated fat consumption results in LDL with less cholesterol, more protein and triglycerides, and a lower ratio of cholesterol to protein (6,30,40). As with the lipoprotein concentration, we observed an effect opposite to that expected. Soybean oil increased cholesterol, decreased protein, and increased the ratio of cholesterol to protein. We observed no effect of protein source on LDL composition, but other workers have shown that animal proteins may increase LDL-cholesterol without changing apo-B (7,41). The ratio of cholesteryl ester to triglyceride in LDL also may be increased by animal protein (9).

The values of the kinetic parameters measured in our pigs are of the same order of magnitude as those reported by other workers. Half-lives of 0.8 to 4.5 hr have been reported for the first exponential and 19.3 to 22.5 hr for the second exponential component for the disappearance of [^{125}I]LDL (42–44). These half-lives would correspond to those for LDL-protein in this study. Fractional catabolic rates measured for [^{125}I]LDL or LDL apo-B ranged from 0.015 to 0.055 per hr (42–45); fractional catabolic rates observed in this study were slightly higher. Irreversible losses in our pigs were greater than the flux rates of LDL apo-B in miniature pigs fed chow (45). It should be noted, however, that considerable variability exists between individual pigs.

Fractional catabolic rates and extent of irreversible loss of both LDL-protein and LDL-cholesterol tended to be less, and the half-life of the second component tended to be greater, in pigs consuming egg white than those consuming soy protein. This observation suggests that the protein source influenced the rate of LDL catabolization; that is, animal protein caused LDL to be removed from the circulation

at a slower rate than did plant protein. Similar observations have been made with rabbits (9,46) and rats (47). Nestel and coworkers, however, found that vegetarians had significantly lower flux of LDL than did omnivorous controls (48).

Dietary fat source had no consistent effect on LDL clearance from plasma in our study, although there was a tendency for soybean oil consumption to decrease the half-life of the second component for LDL-protein and to increase the irreversible loss of LDL-cholesterol. Other studies have shown diets high in polyunsaturated fat to have variable effects (49-51). The influence of fat type on LDL clearance, therefore, remains to be clarified.

Pigs consuming beef tallow in our study had greater plasma cholesterol concentrations, but lesser LDL-cholesterol concentrations and greater HDL to LDL ratios. These results suggest that elevated plasma cholesterol concentration alone may be misleading and emphasize the contention that the distribution of cholesterol in the lipoproteins also must be considered when assessing the effects of a dietary regime on overall atherogenic risk. The observation that dietary fat source influenced LDL concentration and composition, while protein source influenced rate of LDL clearance, suggests that protein and fat influence plasma lipid concentrations by different mechanisms. Perhaps dietary fat exerts its control by changing the cholesterol-carrying ability of plasma through an interaction between LDL concentration and composition, whereas protein has its effects at the level of the tissues by influencing the interaction of LDL with receptors or by altering the extent of receptor-independent catabolism of LDL. Additional study will be necessary to establish definitively the mechanisms whereby dietary protein and fat influence LDL concentrations, composition and clearance and to establish the extent to which diet-induced alterations in these metabolic parameters affect the relative risk of atherosclerosis and coronary heart disease.

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Enhancement of Eicosaenoic Acid Lipoxygenation in Human Platelets by 12-Hydroperoxy Derivative of Arachidonic Acid.

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ABSTRACT

Human platelet lipoxygenase activity toward several eicosaenoic acids was measured in intact cells as well as in subcellular fractions (cytosol and membranes). In whole platelets, the lipoxygenation of eicosaenoic acids was enhanced greatly by high concentrations of aspirin, which partially inhibit the peroxidase activity associated with the pathway. The lipoxygenation also was increased by arachidonic acid (AA) or its lipoxygenase product, 12-hydroxyperoxy-eicosatetraenoic acid (12-HPETE). Similarly, prostanoid precursors, dihomogammalinolenic (DHLA) and eicosapentaenoic (EPA) acids also were better converted by cyclooxygenase in the presence of AA or 12-HPETE. Among the eicosaenoic acids tested, EPA oxygenation was affected most.

Using cytosol or membranes as the lipoxygenase source instead of whole cells led to completely different results. AA exerted a competitive inhibition upon the other eicosaenoic acid oxygenation except that of EPA, for which a dual effect of AA was observed. This makes questionable the use of platelet subfractions for investigating lipoxygenase activity.

We conclude that platelet lipoxygenation of eicosaenoic acids appears peroxide-dependent, especially for apparent poor substrates like EPA. This might be relevant in respect to 12-HPETE, which is the main hydroperoxy derivative to be produced during platelet activation.
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INTRODUCTION

When blood platelets are incubated with aggregating agents, such as thrombin or collagen, endogenous arachidonic acid is liberated from phospholipids and subsequently oxygenated by both cyclooxygenase and lipoxygenase. The oxygenation of arachidonic acid by platelet cyclooxygenase and the biological activity of its products have been described widely (1). Lipoxygenase activity of human blood platelets is associated with a glutathione-dependent peroxidase (2) for the production of 12-hydroxy derivatives from eicosaenoic acids (3-5). This enzyme complex has been localized mainly in cytosol, although a substantial part of the activity was thought to be membrane-bound (6-9). Recently we have shown that the membrane-bound activity was found preferentially in intracellular membranes where both platelet cyclooxygenase and thromboxane synthase are located (9).

On the other hand, a modulating activity of 12-lipoxygenase products upon prostanoid-induced platelet aggregation has been reported (10,11). This effect was observed with derivatives obtained from arachidonic acid as well as from other eicosaenoic acids and appears to be stereospecific (11).

Since different eicosaenoic acids can arise in platelet phospholipids in response to different diets (12), they may interact during their oxygenation with arachidonic acid, the main polyunsaturated fatty acid to be liberated from phospholipids when platelet activation occurs, as we have found previously for eicosapentaenoic acid (13). In the studies reported here, we have investigated the effect of arachidonic acid or some of its lipoxygenase products upon the lipoxygenation of different eicosaenoic acids in intact cells as well as in platelet subfractions.

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EXPERIMENTAL PROCEDURES

Materials

[1-^{14}C]-Arachidonic or AA (55 Ci/mole) and [2-^{14}C]-8,11,14-eicosatrienoic or DHLA (55 Ci/mole) acids were provided from Amersham International, U.K. [1-^{14}C]-5,8,11,14,17-eicosapentaenoic or EPA (50 Ci/mole) acid was purchased from New England Nuclear, Boston, Massachusetts. [1-^{14}C]-5,8,11-eicosatrienoic or 20:3 n-9 (16.5 Ci/mole), its unlabelled form and 5,8,11,14-heneicosatetraynoic acids were provided by H. Sprecher, Columbus, Ohio. The other unlabelled acids were acquired from Sigma, St. Louis, Missouri. Silica gel G plates were obtained from Merck, Darmstadt, Germany Federal Republic. Nucleosil $5C_{18}$ and partisil-5 for high performance liquid chromatography (HPLC) were purchased from Macherey-Nagel, Duren, German Federal Republic, and Whatman, Clif-

ton, New Jersey, respectively. Organic solvents and the various other reagents were obtained from Prolabo, Paris, France.

Platelet Preparation and Fractionation

Venous blood was collected from human volunteers who had not taken any drug for at least 10 days. Platelets were then isolated as described previously (14) and resuspended either into a Tyrode HEPES buffer (THB) (NaCl 136 mM, $NaHCO_3$ 12 mM, NaH_2PO_4 0.41 mM, KCl 4 mM, $MgCl_2$ 1mM, HEPES 5 mM and glucose 5.5 mM) for using intact cells or into a washing buffer pH 7.2 containing NaCl 152 mM, KCl 4 mM, EDTA 3 mM and HEPES 10 mM for further homogenization (9). For the later purpose, platelets were routinely aspirinized by incubation with aspirin (2×10^{-4} M) for 10 min at 37 C, resuspended in a sorbitol buffer giving 3×10^9 platelets/ml (sorbitol 0.34 M, HEPES 10 mM, pH 7.2) and sonicated as previously reported (15). Then the sonicate was centrifuged at 13,000 g for 20 min at 4 C to pellet the granule fraction. The supernatant was removed from above the granule pellet and centrifuged at 100,000 g for 90 min at 4 C to sediment a mixed membrane fraction including surface and intracellular membranes, the latter supernatant representing the cytosol (9). Membranes and cytosol were stored at -70 C without any substantial loss of lipoxygenase activity for one month.

Lipoxygenase Activity Measurements

Assays on intact platelets were conducted with 0.4 ml of platelet suspension (3×10^8 platelets/ml). Four nmoles of each labelled fatty acid (220, 220, 200 and 66 nCi of AA, DHLA, EPA and 20:3n-9, respectively) were incubated in the absence or presence of either unlabelled AA, 12-HPETE, 12-HETE or 15-HPETE for 4 min at 37 C. Incubations were terminated by the addition of three volumes of ethanol, and total lipids were extracted twice with chloroform in the presence of butylated hydroxytoluene (5×10^{-5} M) as an antioxidant (13).

Substrate curves were established with platelet membranes or cytosol. 0.1 Mg (membranes) or 0.2 mg (cytosol) of proteins were incubated in 0.5 ml THB for 1 min at 37 C with the appropriate labelled fatty acid brought in 2 μl of ethanol. The range concentration was 2 to 100 μM for each substrate except for EPA which was studied from 2 to 15 μM. The same incubations also were done in the presence of 10 μM unlabelled AA. Reduced glutathione (0.1 mM with cytosol and 1 mM with membranes) was added as a cofactor of the peroxidase associated to the lipoxygenase pathway (8). Blanks were prepared by boiling lipoxygenase fractions or in the presence of 5,8,11,14-heneicosatetraynoic acid, a specific 12-lipoxygenase inhibitor (16). Incubations were terminated by acidification to pH 3 with 1 M HCl, immediately followed by the extraction of the substrates and their products by 5 ml of cold diethylether. The resulting organic phase obtained by centrifugation was reduced under vacuum and residual water removed azeotropically by adding dry ethanol.

Analysis of Lipoxygenase Products

Lipid residues redissolved in ether were submitted to thin layer chromatography (TLC) on Silica Gel G. The plates were developed with the hexane/diethylether/acetic acid (60:40:1, v/v/v) mixture. In the case of short incubations with platelet subfractions, the TLC was performed immediately at 4 C to avoid the degradation of labile compounds, e.g. hydroperoxy derivatives and hydroxy-epoxy compounds, during the separation. Then a quantitative radiochromatogram was done (13). A typical radiochromatogram is shown in Figure 1. Hydroxy derivatives of C_{17} acids providing through the cyclooxygenase pathway and thromboxanes were quantified from intact platelets as described previously (13). Briefly, total radioactivity was considered as representing the initial amount of labelled substrate. The integrated peaks then calculated as percentage of total radioactivity could be quantitated in nmoles of products. When platelets subfractions were used, peaks I, II and III (Fig. 1) were totaled to represent lipoxygenase products because platelets were pretreated with 2×10^{-4} aspirin.

Preparation of 12-HPETE, 12-HETE and 15-HPETE

12-HPETE was prepared by incubating platelet homogenates with 2.5×10^{-4} M AA for 5 min in the presence of 2×10^{-3} M aspirin. This aspirin concentration allowed a partial inhibition of the peroxidase associated with the lipoxygenase pathway (17). The reaction was terminated by acidification to pH 3, and acidic derivatives were extracted by cold diethylether as described above. After a TLC separation of 12-HPETE from the excess of substrate, it was purified further by straight phase HPLC using a 250 x 4.6 mm column packed with Partisil-5. It was eluted in the fraction 16 to 20 ml of hexane/isopropanol/acetic acid (99:1:0.1, v/v/v) (12-HETE being eluted in the fraction 11-13 ml). 12-HETE and 15-HPETE were prepared in using platelet and soybean lipoxygenases, respectively, and they were purified as described previously (11,18). The three compounds were quantitated by UV absorption at 235 nm assum-

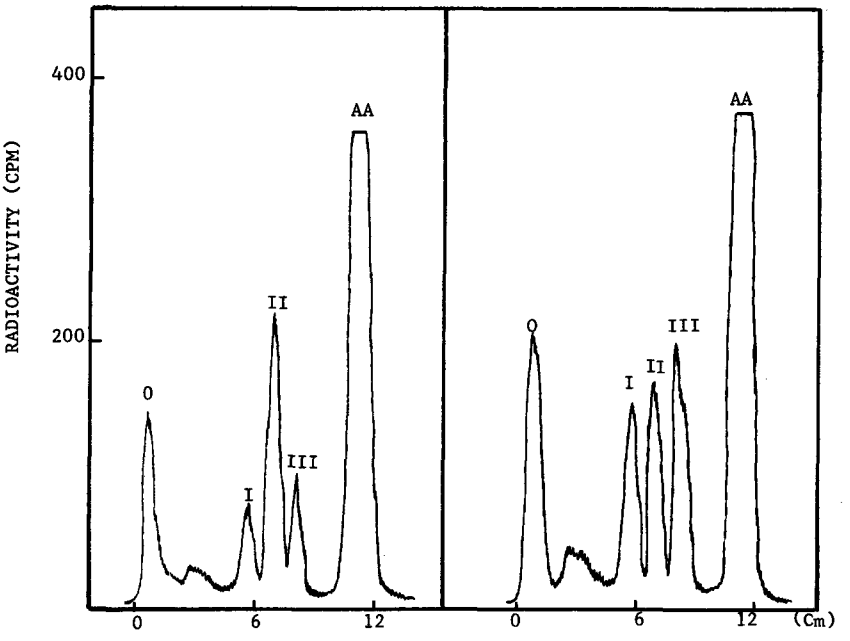

FIG. 1. Typical radiochromatogram of the extract providing from platelet cytosol (left panel) or membranes (right panel) incubated with [1-^{14}C]arachidonic acid (10^{-4} M, 200 nCi) in Tyrode buffer at 37 C for 1 min in the presence of 2.10^{-4} M aspirin. The products were extracted by cold diethylether and TLC plates developed as described in experimental methods. O, origin; I, presumably 10-OH-11, 12-epoxy-eicosatrienoic acid; II, 12-HETE; III, 12-HPETE; AA, remaining arachidonic acid.

ing an ϵ of 3×10^4 and stored in absolute ethanol at -70 C under nitrogen.

Protein Determination

Protein concentration of platelet subfractions was measured by a microtannin turbidimetric method using bovine serum albumin as standard (19).

Statistics

Paired t-test was used to compare the formation of lipoxygenase products in intact cells. Linear regression was performed in the Lineweaver-Burk representation of the substrate curves.

RESULTS

Oxygenation of Eicosaenoic Acids by Intact Human Platelets

The oxygenation of EPA by platelet lipoxygenase, which previously has been found markedly increased by AA, was studied further in this respect. The formation of its 12-hydroxy derivative was measured after 4 min incubation of 10^{-5} M EPA in the absence or presence of 2×10^{-3} M aspirin. The effect of either AA, 12-HPETE, 12-HETE or 15-HPETE also was tested. Table 1 shows a strong potentiation of the 12-hydroxide formation from EPA by AA or 12-HPETE but no effect of 12-HETE or 15-HPETE. Besides, the high concentration of aspirin used in this set of experiments further

TABLE 1

Effect of AA or Some of its Lipoxygenase Products upon the Lipoxygenation of EPA in the Absence (A) or Presence (B) of a High Concentration of Aspirin (2×10^{-3} M)

	A	B	Ratio B/A
Control	0.28 ± 0.09	2.3 ± 0.68	8.2
AA (10^{-5} M)	3.8 ± 2.1	10.3 ± 3.2	2.7
12-HPETE (5×10^{-6} M)	4.7 ± 1.2	8.5 ± 1.9	1.8
12-HPETE (5×10^{-6} M)	1.9 ± 0.2	7.5 ± 1.6	3.9
12-HETE (5×10^{-6} M)	0.23 ± 0.07	–	–
15-HPETE (5×10^{-6} M)	0.31 ± 0.15	1.7 ± 0.10	5.4

Results in nmoles/10^9 platelets (n=2) represent the mean ± range of the formation of the hydroxy derivative of 10^{-5} M EPA after 4 min incubation.

increased the lipoxygenase product synthesis from EPA, even when it was already enhanced by AA or 12-HPETE. Partial inhibition of the peroxidase activity by high concentrations of aspirin (2×10^{-3} M) also resulted in a marked potentiation of lipoxygenase activity toward AA, DHLA or 20:3n-9 (Table 2). However, enough peroxidase activity still remained because no 12-hydroperoxide could be measured after 4 min incubation. An increased formation of hydroperoxides could be visualized only on TLC plates in the first 30 sec incubation (results not shown). In contrast, preincubation with aspirin (2×10^{-4} M), which only blocked the cyclooxygenase, did not modify the lipoxygenase product formation.

TABLE 2

Effect of Aspirin on the Lipoxygenation of Various Eicosaenoic Acids

	Aspirin		
	0	2×10^{-4} M	2×10^{-3} M
AA	2.35 ± 0.55	2.45 ± 0.65	10.3 ± 2.5
EPA	0.32 ± 0.10	0.43 ± 0.13	2.1 ± 0.5
DHLA	0.85 ± 0.43	0.42 ± 0.03	4.4 ± 3.1
20:3n-9	0.75 ± 0.65	0.71 ± 0.15	5.3 ± 3.2

Results in nmoles/10^9 platelets represent the mean ± S.D. of three experiments.

Each acid was incubated for 4 min at 10^{-5} M.

The effect of AA or 12-HPETE upon the oxygenation of other eicosaenoic acids also was investigated. Figure 2 shows the formation of 12-hydroxy derivatives from either AA, EPA, DHLA or 20:3n-9 through the lipoxygenase pathway. While AA (10^{-5} M) was the best substrate of platelet lipoxygenase (the formation of 12-hydroxy derivatives from EPA, DHLA and 20:3n-9 incubated alone being 11, 24 and

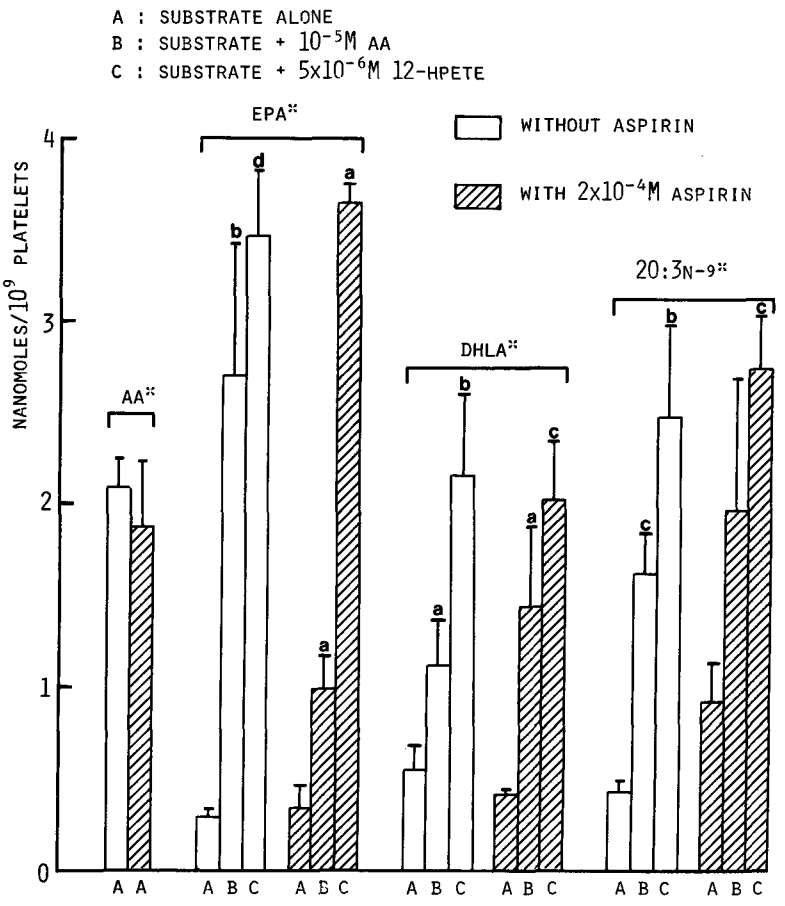

FIG. 2. Effect of AA or 12-HPETE upon the lipoxygenation of EPA, DHLA or 20:3n-9 in the absence or presence of aspirin (2×10^{-4} M). Results represent the mean ± SE of at least four experiments. a, $p<0.05$; b, $p<0.02$; c, $p<0.01$; d, $p<0.005$, compared to control (substrate alone).

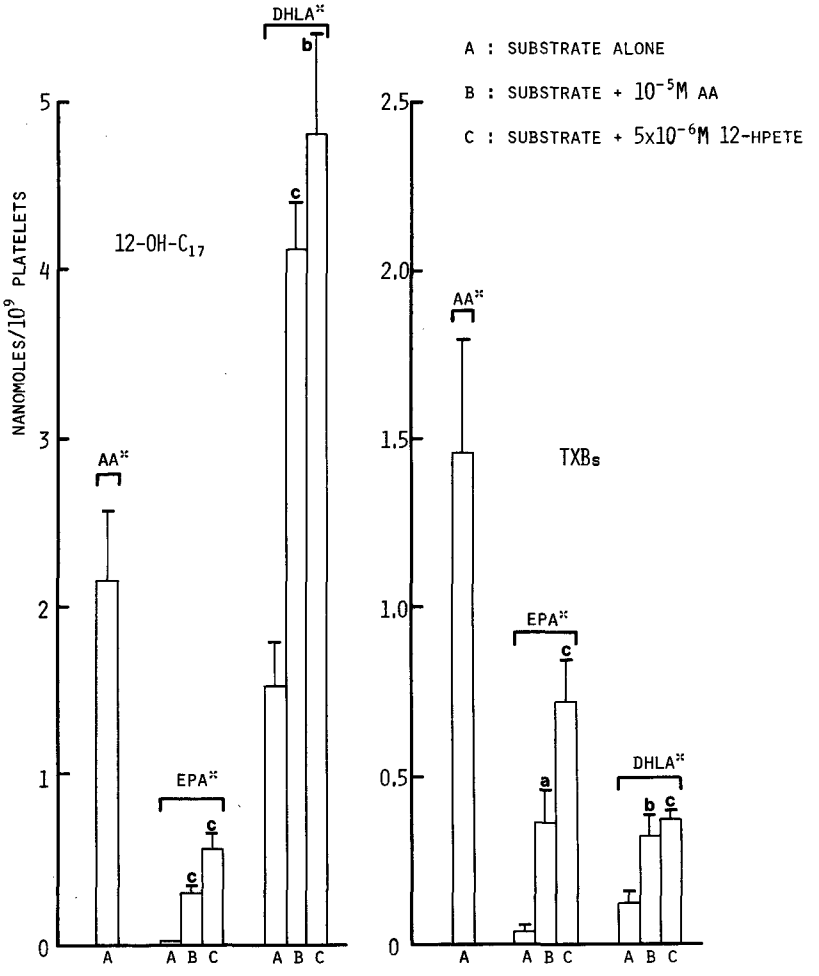

FIG. 3. Effect of AA or 12-HPETE upon the formation of cyclooxygenase products (hydroxy-heptadecaenoic acids: 12-OH-C_{17} and thromboxanes: TXBs) on EPA or DHLA. Results represent the mean ± SE of four experiments. a, $p<0.05$; b, $p<0.02$; c, $p<0.01$, compared to control (substrate alone).

33% of that of 12-HETE, respectively) the presence of 10^{-5} M AA, or 5×10^{-6} M 12-HPETE added simultaneously to EPA, DHLA or 20:3n-9 markedly and significantly increased their transformation. This stimulated formation of 12-hydroxides was then equal to that of 12-HETE and even greater than that from EPA. In agreement with Table 2, 2×10^{-4} M aspirin, which is assumed to inhibit cyclooxygenase solely, did not significantly affect the 12-hydroxy-eicosaenoic acids produced from each precursor whatever the conditions tested. 12-Hydroxy-heptadecaenoic acids and thromboxanes produced from EPA and DHLA were similarly enhanced by AA or 12-HPETE (Fig. 3).

Lipoxygenase Activity of Platelet Subfractions Toward Eicosaenoic Acids

When cytosol or membranes were used as the lipoxygenase source, several products appeared with AA as substrate (Fig. 1). Apart from the remaining substrate, three compounds could be identified: 12-HPETE (R_f 0.45), 12-HETE (R_f 0.37) and a more polar component co-migrating with a standard HHT (R_f 0.30), presumably corresponding to 10-hydroxy-11, 12-epoxy-eicosatrienoic acid. A second elution of the plate with a more polar mixture, diethylether/methanol/acetic acid (90:1:2, v/v/v), revealed trace amounts of trihydroxy-eicosatrienoic acids. Similar radioactive patterns were obtained by

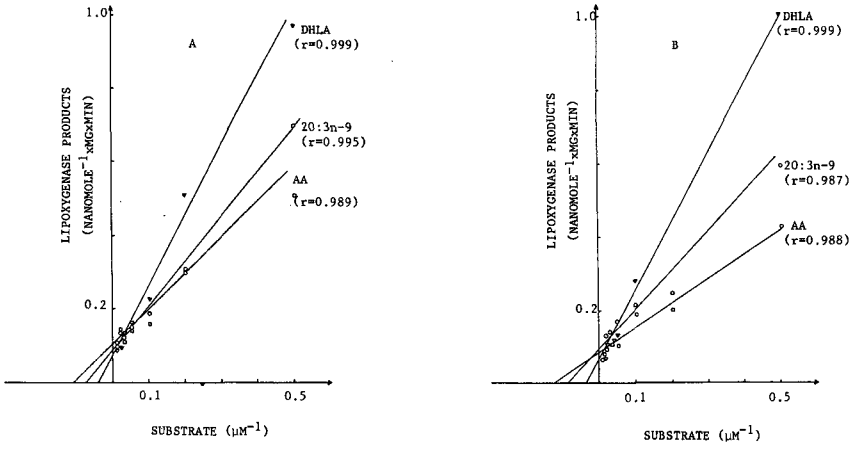

FIG. 4. Double reciprocal representation of the lipoxygenase kinetic toward AA, DHLA or 20:3n-9. Data were obtained with platelet cytosol (A) or membranes (B).

TABLE 3

Lipoxygenase Activity in Platelet Subfractions

Substrates	Range concentration (10^{-6} M)	Cytosol		Membranes	
		Apparent Km (10^{-6} M)	Vm nmoles/ min/mg prot	Apparent Km (10^{-6} M)	Vm nmoles/ min/mg prot
20:4n-6	2-100	7	12	8.5	19
20:3n-9	2-100	12	12	14.5	16
20:3n-9 + 10^{-5} M 20:4n-6	2-100	22	8.5	28	15.5
20:3n-6	2-100	21.5	16	24	23
20:3n-6 + 10^{-5} M 20:4n-6	2-100	58	15	66	25
20:5n-3	2-15	6.5	5	7	7

Results represent the mean of two determinations.

using EPA, 20:3n-9 or DHLA as substrates. The lipoxygenase activity therefore was measured as the sum of hydroperoxide, hydroxide and hydroxy-epoxide produced from each radiolabelled substrate. Substrate curves concerning AA, 20:3n-9 and DHLA were performed from 2 to 100 μM. While these curves were substrate-saturated, that concerning EPA showed a decreased formation of its 12-hydroxy derivative over 15 μM (results not shown). For this reason, the substrate curve of the lipoxygenation of EPA was performed between 2 and 15 μM. Figure 4 gives an example of a double reciprocal representation for the conversion of AA, 20:3-n-9 or DHLA by cytosolic and membrane-bound lipoxygenases. Although the measurements resulted from both lipoxygenase and peroxidase activities, apparent Vmax and Km may be considered as referred to lipoxygenase activity, all the products of the reaction being measured. Then, the lipoxygenase activities measured in both platelet subfractions were similar in respect to the substrate. In each subfraction, while the Vmax values were not much different, apparent Km values revealed a highest affinity for AA followed by 20:3n-9. This is confirmed by the data of Table 3 representing the mean of two different experiments. The effect of 10^{-5} M AA was also tested at each substrate (20:3n-9 or DHLA) concentration. As shown in Table 3, AA did not affect the Vmax very much, and it markedly increased the apparent Km. This suggests a competitive inhibition of AA upon the oxygenation of the other substrates. The oxygenation of EPA, which was peculiar as mentioned above, presented an apparent Km comparable to that of AA with a lower Vmax. The effect of 10^{-5} M AA on EPA lipoxygenation

was quite atypical with an inhibition until 5 μM EPA and a stimulation over 10 μM. Finally, all the data were comparable with either platelet subfraction although membranes, as the enzymic source, gave the highest Vmax values.

DISCUSSION

In the present work, the effect of AA and its lipoxygenase products upon the oxygenation of other eicosaenoic acids has been investigated. We confirm our previous studies showing that AA strongly potentiates the oxygenation of EPA (13). Further investigations of this effect revealed that the increased EPA lipoxygenation could be reproduced by the 12-hydroperoxy derivative from AA but not by its reduced product, the 12-hydroxy derivative. This confirms a recent report (20). The potentiation by 12-HPETE seemed to be dose-dependent although not directly related. However, this point might be difficult to appreciate, the catabolism of 12-HPETE by platelet glutathione-peroxidase being assumed to be very active. In addition, we found that 15-HPETE did not share the potentiating activity of 12-HPETE. If we argue that 12-HPETE acts in bringing peroxides needed for the oxygenation of EPA as it was first hypothesized (21), the absence of potentiating activity of 15-HPETE could be interpreted as the effect of the increased EPA lipoxygenation and the inhibition of platelet 12-lipoxygenase by 15-lipoxygenase products as described earlier (22). Besides, the conversion of DHLA and 20:3n-9 by lipoxygenase of intact platelets also was increased by either AA or 12-HPETE, although less than observed for EPA, indicating that 12-HPETE could activate the lipoxygenation of any polyunsaturated fatty acid.

In the presence of high concentrations of aspirin (2×10^{-3} M), known to partially inhibit the peroxidase associated with the lipoxygenase pathway (17), transformation of EPA by this enzyme was greatly enhanced. The lower the EPA oxygenation, the higher was the enhancement by aspirin. This fact is in good agreement with the peroxide requirement of the EPA oxygenation. Moreover, the lipoxygenation of other eicosaenoic acids also was potentiated when high concentrations of aspirin were present, suggesting that this potentiation was due to their own hydroperoxides. This already was reported for AA where 12-HPETE enhanced its own formation (23). In contrast, using 2×10^{-4} M aspirin, a concentration believed not to alter the peroxidase activity of the lipoxygenase pathway (17), the lipoxygenation of eicosaenoic acids was not significantly increased. This suggests that the cyclooxygenase products do not interfere very much with the lipoxygenase activity, although a previous work reported that oral administration of aspirin induces a lag phase in the conversion of arachidonic acid into 12-HETE, indicating a positive effect of peroxides brought by PGG_2 upon the lipoxygenation (24).

Our results concerning the conversion of EPA and DHLA by the cyclooxygenase activity also revealed a potentiation by AA or 12-HPETE. Again, the potentiation was most important with EPA as precursor because its two main products of this pathway were very weakly produced (TXB_3) or even undetectable (12-hydroxy-heptadecatetraenoic acid) when it was incubated alone with platelets. These data confirm the requirement of platelet oxygenases for peroxides, this requirement being essential for the EPA oxygenation.

The situation was quite different when platelet subfractions, instead of intact platelets, were used as a lipoxygenase source. Supplementary reduced glutathione was necessary in the incubate for producing consistent amounts of hydroxy derivatives. However, hydroperoxides and their by-products hydroxy-epoxides still were detected. With either the cytosol or membranes, AA remained the best substrate, considering the ratio Vm/Km as an index of efficiency. At the opposite of intact platelets, platelet subfractions consistently oxygenated EPA, at least in the 2-15 μM range. Our data are in agreement with those obtained with a semi-purified cytosolic lipoxygenase of bovine platelets (6) and an acetone powder lipoxygenase providing from human platelets (4). In the latter case, the range concentration used for determining the Km of the EPA fransformation was also much lower than for other substrates, suggesting that the peculiar behavior of EPA toward its lipoxygenation appears whatever the lipoxygenase preparation.

In contrast to that observed in intact platelets, AA did not enhance the oxygenation of the other eicosaenoic acids. AA competitively inhibited the transformation of 20:3n-9 or DHLA. These results suggest that enough peroxides were present in the incubates, allowing a consistent lipoxygenation of every substrate. The origin of such peroxides is unclear. They might be generated during cell fractionation and/or by altering the glutathione peroxidase activity. Under these conditions, peroxides brought by AA could be unnecessary and AA might act only as a competitive inhibitor by structural analogy. The effect of AA upon the EPA lipoxygenation in both platelet subfractions is much more complicated to interpret. The inhibition observed below 5 μM EPA could be due to a similar competitive effect of AA. At

higher concentrations of EPA, where its lipoxygenation felt down, the potentiating effect of AA might still reveal a peroxide requirement. Comparing intact platelets and platelet subfractions, we could argue that in the presence of unlabelled AA, the other eicosaenoic acids studied would be less incorporated into phospholipids of intact platelets as described elsewhere (25). This would increase their availability for oxygenases and change the behavior of whole cells and subfractions which do not properly acetylate, fatty acids. This is unlikely under our conditions because only EPA acylation into phospholipids was decreased by unlabelled AA (26). Besides, increasing concentrations of EPA did not enhance its oxygenation much (13).

Finally, the comparison between cytosolic and membrane sources of lipoxygenase confirms our previous findings for the AA lipoxygenation (9). The enzyme activities were similar on several points although the Vm values were always higher in membrane preparations than in cytosol. This reinforces the assumption already made that the membrane-bound lipoxygenase might be more efficient than the soluble form, at least at the early stages of the oxygenation (9).

In summary, we may underline that under physiological conditions (intact platelets), eicosaenoic acids and particularly EPA need a certain peroxide tone to be oxygenated by lipoxygenase. AA, which is the major polyunsaturated fatty acid to be liberated from platelet phospholipids when cell activation occurs, then appears as the main furnisher of peroxides, through 12-HPETE. Using platelet subfractions masks the peroxide requirement, presumably because of the presence of enough peroxides in the preparations, and then makes questionable this approach for investigating platelet lipoxygenase activity.

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Effect of Dietary Restriction on the Plasma Apolipoprotein Pattern in Cholesterol-fed Rabbits

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ABSTRACT

Dietary restriction (half of the control ration) was performed in rabbits given either standard or cholesterol-rich diets. The plasma apolipoproteins were studied on the total, d < 1.21, lipoprotein fraction using polyacrylamide gel electrophoresis. A marked rise in the amount of both apo-B and apo-E appeared in cholesterol-fed rabbits and was enhanced by dietary restriction. These results reflect the aggravation of hypercholesterolemia when cholesterol feeding is carried out in underfed rabbits. In all groups only one molecular species of apo-B, identified as apo-B-100, was present after overnight fasting. Thus, lipoproteins which accumulate in the plasma following cholesterol feeding, associated with dietary restriction or not, probably are remnants of hepatogenous triglyceride-rich lipoproteins.
Lipids 20:751-756, 1985.

INTRODUCTION

Cholesterol-fed rabbits have been used extensively as a model for the study of experimental hypercholesterolemia and the related alterations in plasma lipoproteins (1-4). Previously we showed that the effect of cholesterol feeding could be markedly aggravated by a reduction of the total dietary intake. The rise in plasma cholesterol was 2-fold higher in rabbits on restricted diets than in rabbits on normal caloric ration, and this excess in circulating cholesterol was carried mainly by d < 1.006 lipoproteins (5). In addition, the adverse effects of dietary restriction, when associated with cholesterol feeding, resulted in more extensive atherosclerosis (5).

The aim of the present study was to define the origin of the additional lipoproteins which accumulate in underfed rabbits. This was investigated by the study of the different molecular species of apo-B. The fact that the apo-B synthesized in the liver (B-100) differs in apparent molecular weight and amino acid composition from apo-B of intestinal origin (B-48) recently has been demonstrated in humans (6). Similar heterogeneity exists in rabbits (7,8) and, as shown by perfusion techniques, in this species the liver secretes only apo-B-100 (8). Therefore, the proportion of the two forms of apo-B should reflect the circulating intestinal and hepatic particles.

Data on changes in the apolipoprotein levels also are reported.

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MATERIALS AND METHODS

The animals were male New Zealand rabbits of mean weight 2.4 kg at the beginning of the experiment. Standard rabbit diet and the same diet supplemented with cholesterol were provided by UAR (Villemoisson/Orge). The rabbits were given the following daily dietary ration: Control (C), 160 g of standard diet; Restricted Control (RC), 80 g of standard diet; Cholesterol (Ch), 160 g of standard diet + 0.2 g/kg body weight of cholesterol; Restricted Cholesterol (RCh), 80 g of standard diet + 0.2 g/kg body weight of cholesterol. Cholesterol intake was adjusted to body weight using diets containing 5% cholesterol with which the daily amount necessary to provide 0.2 g/kg was calculated for each rabbit. The dietary ration was then complemented with standard diet.

During the experiment, body weight and plasma cholesterol were measured weekly. The measurement of plasma cholesterol (9) was carried out after overnight fasting.

After five weeks on the respective diets the animals were killed by exsanguination following an overnight fast. Blood was collected on EDTA (0.1%), chloramphenicol (0.005%), streptomycin (0.01%) and sodium azide (0.02%). Plasma was separated immediately by centrifugation and 10 μl/ml PMSF added (0.1 M in isopropanol). Plasma density was adjusted to 1.21 with KBr, and total lipoproteins were isolated by ultracentrifugation at 45,000 rpm for 28 hr in a Beckman 50.2 Ti rotor. The lipoprotein fraction was then dialyzed against

0.15 M NaCl containing antibiotics, azide and EDTA.

The concentration of apo-B was determined following isopropanol precipitation according to Holmquist et al. (10). Total proteins of the $d < 1.21$ lipoproteins and isopropanol-soluble protein were measured by the method of Lowry et al. (11), and the apo-B concentration was calculated from the difference. In order to test the efficiency of precipitation of rabbit apo-B by isopropanol, supernatant samples were analyzed by SDS polyacrylamide gel electrophoresis (12). No detectable band of apo-B was seen in 3% acrylamide gels. In addition, no changes appeared in the proportion of the other apolipoproteins when separated in 12% acrylamide gels.

The concentration of the other apolipoproteins was estimated using 0.1% SDS, 12% polyacrylamide gel electrophoresis of the total, $d < 1.21$, lipoprotein fraction. Electrophoresis was carried out after rapid delipidation by diethyl-ether and 1 min of incubation at 90 C in 2% SDS, 5% 2-mercaptoethanol. All samples were run in duplicate with an increased amount of protein the second time. The proportion of each apolipoprotein, determined by scanning using a SEBIA densitometer, was multiplied by the isopropanol soluble protein concentration to calculate the apolipoprotein plasma concentration. Although variation in protein chromogenicity may limit this technique of apolipoprotein quantification, it is valid for comparison between groups.

Molecular species of apo-B were analyzed after separation by electrophoresis in 3 or 3.5% polyacrylamide gel containing 0.1% SDS. For each sample, at least two and sometimes more migrations were carried out with increasing amounts of proteins applied to the gel. A control experiment was carried out with samples of rabbit chylomicrons, VLDL, LDL and HDL subjected to similar analysis. Chylomicrons were obtained from a rabbit fed a diet supplemented with olive oil three days prior the collection; they were separated by centrifugation and two additional purifications at density 1.006 (15,000 rpm for 30 min). The other lipoproteins were then separated on a KBr density gradient according to Redgrave et al. (13).

RESULTS

The mean (±SEM) changes in body weight during the experiment were 420 ± 23 g increase and 98 ± 19 g loss for rabbits on normal and restricted caloric ration, respectively.

Dietary cholesterol induced a rapid rise in plasma cholesterol which was more pronounced in rabbits on restricted diets. No changes in cholesterolemia were observed when dietary restriction was not associated with cholesterol supplementation of the diet (Fig. 1).

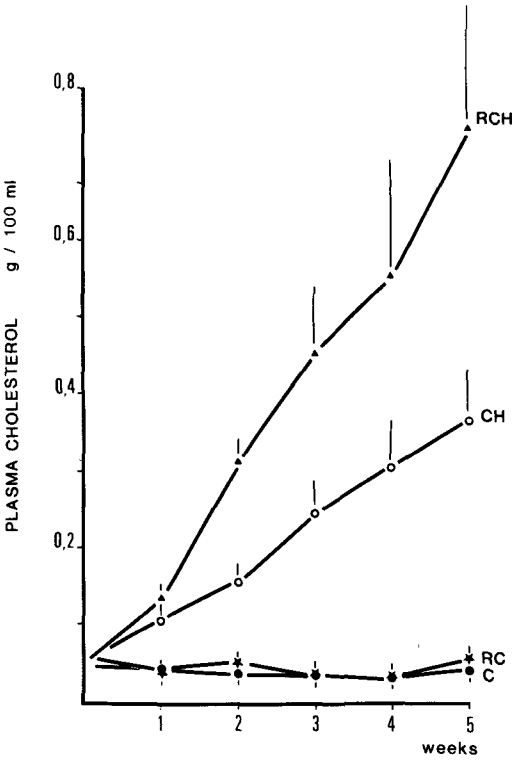

FIG. 1. Influence of dietary restriction on plasma cholesterol in rabbits fed either standard or cholesterol rich diets. C, control; RC, restricted control; Ch, cholesterol; RCh, restricted cholesterol. Values are mean ±SEM of 7 rabbits.

The apolipoprotein patterns of the total, $d < 1.21$, lipoprotein fraction observed on 12% SDS polyacrylamide gel electrophoresis are shown in Figure 2. The different apolipoproteins were identified by comparison of their apparent molecular weights with those of human apolipoproteins. In the control rabbits, the major apolipoprotein other than apo-B was apo-A_I. Both groups receiving cholesterol showed a pattern differing from that of the control with a large increase in apo-E. The values of the plasma concentration of the different apolipoproteins are presented in Figure 3. The estimated level of total apo-E increased from control values by 4.5 and 8 times in the cholesterol and restricted cholesterol groups, respectively. A slight increase in the level of apo-A_I also was observed in the restricted

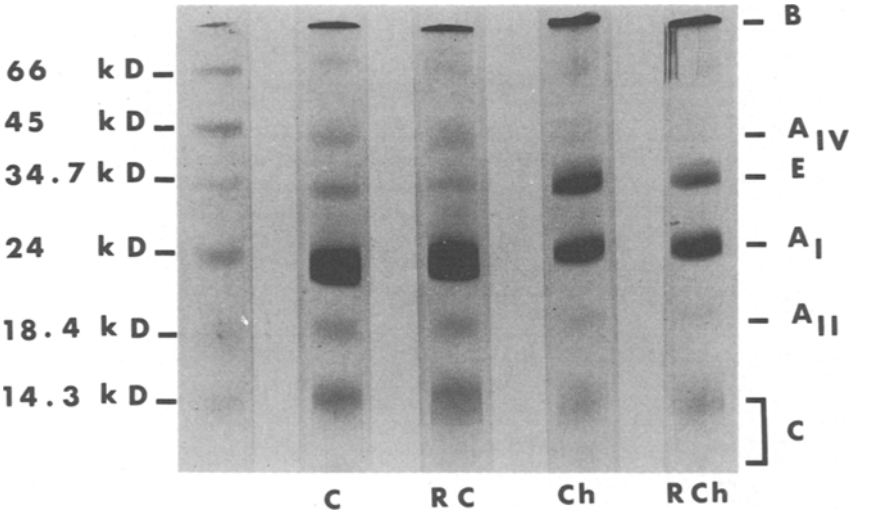

FIG. 2. SDS polyacrylamide gel (12%) electrophoresis of d < 1.21 lipoproteins from control (C), restricted control (RC), cholesterol (Ch) and restricted cholesterol (RCh) rabbits. The mass of protein was 40, 37, 52 and 48 μg, respectively. The different components have estimated molecular weights of 44,000 (apo A_{IV}); 35,300 (apo E); 26,000 (apo A_I); 19,600 (apo A_{II}); 14,300 and 10,400 (apo C). Molecular weight markers were from top bovine albumin, ovalbumin, pepsin, trypsinogen, β lactoglobulin and lysosyme (gel on left).

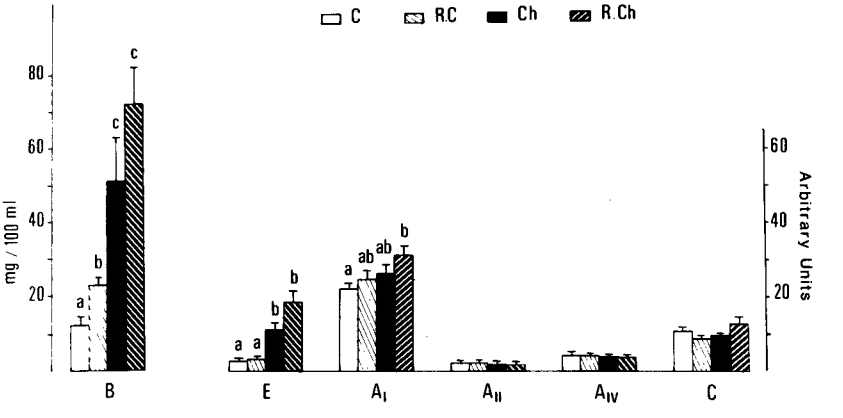

FIG. 3. Influence of dietary restriction on plasma apolipoprotein concentration in rabbits fed either standard or cholesterol-rich diets. C, control; RC, restricted control; Ch, cholesterol; RCh, restricted cholesterol. Values are mean ±SEM of 7 rabbits. a, b, c indicate intergroup statistical differences, means not sharing a common letter are significantly different ($P < 0.05$).

cholesterol group. No more changes appeared in the concentration of the other isopropanol-soluble proteins. Marked changes were found in the level of apo-B following cholesterol feeding, and they were amplified when associated with dietary restriction (4- and 6-fold higher than in controls for cholesterol and restricted cholesterol groups, respectively). Dietary restriction, with the standard cholesterol-free diet, induced a 2-fold increase of apo-B in rabbits on standard diet.

In order to evaluate the proportion of the different forms of apo-B and the possible changes with diet, the total, d < 1.21, lipoprotein fractions also were analyzed by 0.1% SDS, 3.5% polyacrylamide gel electrophoresis as shown in Figure 4. In all groups, only one molecular species was present. The apparent molecular

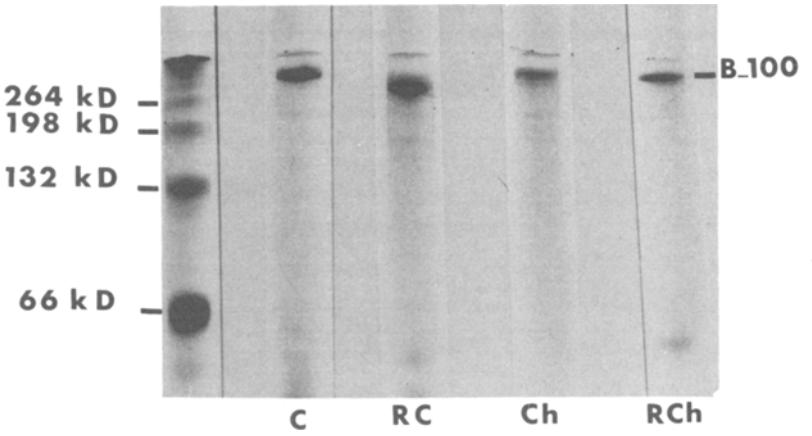

FIG. 4. SDS polyacrylamide gel (3.5%) electrophoresis of d < 1.21 lipoproteins from control (C), restricted control (RC), cholesterol (Ch) and restricted cholesterol (RCh) rabbits. The mass of protein was 25, 28, 5 and 5 μg, respectively. Molecular weight marker was cross linked albumin (gel on left).

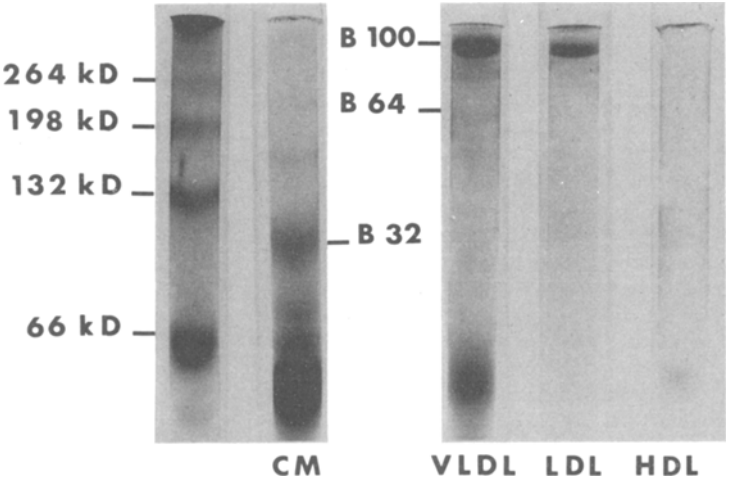

FIG. 5. SDS polyacrylamide gel (3.5%) electrophoresis of chylomicrons (CM), VLDL, LDL and HDL from a normal rabbit. The mass of protein was 80, 53, 17 and 40 μg, respectively. Molecular weight marker was cross linked albumin (gel on left).

weight calculated from 3 or 3.5% polyacrylamide gel electrophoresis was 342,000. In some cases, a minor component (2-3%) of an apparent molecular weight of 220,000 was seen.

The pattern obtained for samples of chylomicrons, VLDL, LDL and HDL from normal rabbit is shown in Figure 5. The component of 342,000 was found in VLDL and LDL, a minor band of 220,000 also was detected in these fractions. In contrast, chylomicrons contained a different molecular species of 110,000. According to Kane (6), the form of higher molecular weight could be referred to as B-100, the others corresponding to B-64 and B-32.

DISCUSSION

Cholesterol feeding is well known to result in a large increase in d < 1.006 lipoproteins of abnormal composition as reported in rabbits (1-3) and various other species (14,15). These lipoproteins, referred to as beta VLDL, are characterized by a dramatic increase in their relative content of cholesterol esters and of apo-E. The high level of apo-E-enriched material resulted in an elevation of the total plasma apo-E concentration accompanying that of apo-B as observed in the present study in cholesterol-fed rabbits. A similar concomitant but consistently higher rise in both apo-B and apo-E appeared in

cholesterol-fed rabbits on restricted diet. These results are in agreement with our previous observations that an additional increase in d < 1.006 lipoproteins occurs when cholesterol feeding is associated with dietary restriction (5). On the other hand, dietary restriction without cholesterol supplementation induced a significant rise in the plasma level of apo-B, but not of apo-E, reflecting the 2-fold increase of LDL previously observed in these experimental conditions (5). In several situations VLDL and IDL from hypercholesterolemic plasma showed an increased level of apo-E. This was reported in nutritional hypercholesterolemia (1-3, 14-17) as well as with genetic defects of the LDL receptor (7). Metabolic studies of apo-E carried out in the rabbit showed that the elevation of the total plasma apo-E concentration following cholesterol feeding resulted partly from overproduction of this apolipoprotein (18,19). Taken together, these observations stress the importance of apo-E in hypercholesterolemia, but the definite role of these changes in apo-E metabolism has not yet been elucidated.

An unexpected observation in this study was the presence of only one molecular species of apo-B in the plasma, whatever the dietary treatment. The heterogeneity of apo-B was first described in humans (6) and rats (20), and it was demonstrated that in humans the apo-B synthesized in the liver (B-100) differs in apparent molecular weight and amino acid composition from apo-B of intestinal origin (B-48) (6). The existence of a similar heterogeneity also was observed in rabbits (7,8). Moreover, in this species as in humans, the liver secretes only the higher molecular weight form of apo-B (B-100) (8). In our experiment the apparent molecular weight of the sole form of apo-B remaining in the plasma after overnight fasting was 342,000. The same component of 342,000 was seen in both VLDL and LDL fractions. This value is very close to the 340,000 reported for apo-B-100 in the rabbit (17,21) and to the 335,000 in the rat (20). Several samples contained trace amounts of 220,000 (B-64), which appeared whatever the dietary treatment. This component, which also was detected in our VLDL and LDL preparations and previously observed in rabbit VLDL, IDL and LDL (21), appears to be different from the intestinal apo-B because chylomicrons contain essentially a protein corresponding to B-32. Therefore, the presence of B-64 may be the result of a particular sensitivity of rabbit apo-B-100 to proteolysis, in spite of the addition of PMSF and antibiotics, as suggested for human apo-B-100 (6).

The fact that we failed to detect any trace amount of the intestinal component (B-32) after overnight fasting indicates normal hepatic removal of chylomicron remnants in both groups receiving dietary cholesterol; thus, the lipoproteins which accumulate in the plasma appear to be of hepatic origin. The hypothesis that in cholesterol-fed rabbits beta VLDL corresponds to chylomicron remnants was first proposed by Ross et al. (22). During the course of our experiment, results obtained in the same laboratory demonstrate that in fact chylomicron remnants are removed equally well by perfused livers of normal and cholesterol-fed rabbits, while the removal of VLDL is impaired in livers of cholesterol-fed rabbits (23). Therefore, the increase in beta VLDL cannot be accounted for by the accumulation of chylomicron remnants. The present study strongly confirms this observation. In contrast, the situation appears to be quite different in cholesterol-fed dogs where the beta-VLDL are composed of chylomicron remnants and lipoproteins of liver origin (24).

It is well established now that the hepatic catabolism of triglyceride-rich lipoprotein is mediated by two distinct types of receptor: the chylomicron remnant receptor and another identical to the LDL receptor first described in fibroblasts (25). In the cholesterol-fed rabbit, the defective hepatic uptake of beta-VLDL was shown to result from saturation and suppression of hepatic lipoprotein receptors analogous to the LDL receptors (26). The careful study of Angelin et al. (27) demonstrates that LDL receptors respond to the changes in cholesterol content of hepatic cells, while chylomicron remnant receptors do not appear to be regulated. Several situations confirm the duality of hepatic receptors and of their regulation mechanisms. In all cases manipulations that affect hepatic LDL receptors do not influence the hepatic uptake of chylomicron remnants (28, 29). Thus, our results suggesting the hepatic origin of the d < 1.006 lipoproteins which accumulate in the plasma following cholesterol feeding are consistent with the recent advances made in the understanding of hepatic lipoprotein receptors.

According to Ross et al. (22), the cholesterol-fed rabbit was thought to be a model for type III hypercholesterolemia. In fact in this disease the fraction with beta mobility contained both the B-48 and the B-100 proteins. Thus, remnant particles appeared to originate from both chylomicrons and hepatic VLDL (30). The finding that probably none of the accumulated lipoproteins in the cholesterol-fed rabbit are derived from the intestine indicates major differences from type III hyperlipoproteinemia.

Our main interest in this study was whether

or not the changes in apolipoproteins following cholesterol feeding were similar in rabbits on restricted or normal caloric ration. The results show that, qualitatively, dietary restriction caused no further changes to occur; the additional increase in plasma cholesterol also appears to be carried by hepatogenous lipoproteins. Nevertheless, the mechanisms involved in the different responsiveness to dietary cholesterol between rabbits on normal or restricted diets remains unknown.

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Cholesterol Transport and Uptake in Miniature Swine Fed Vegetable and Animal Fats and Proteins. 2. LDL Uptake and Cholesterol Distribution in Tissues[1]

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ABSTRACT

In a 2 × 2 factorial arrangement, miniature pigs were fed four diets containing vegetable protein /fat (soybean) and animal protein (egg white)/fat (beef tallow) to demonstrate the effects of protein and fat source on tissue cholesterol concentrations, uptake of intact low density lipoproteins (LDL) and free cholesterol exchange from LDL to tissues. Soybean oil feeding, compared with beef tallow feeding, resulted in greater concentrations of cholesterol in aorta, heart, and large and small intestines. Similar trends were seen in liver, adipose tissue and skeletal muscle. Dietary protein source had little or no effect on tissue cholesterol concentrations. Uptake of intact LDL, as measured by using [^{14}C]sucrose-LDL, tended to be greater in pigs fed soybean oil or soy protein. Net exchange of free cholesterol from LDL, as measured with [^{3}H]cholesterol, tended to be greater when vegetable products were fed. Relative contributions of whole tissues to total uptake by either mechanism were not influenced by diet. Mechanisms in addition to uptake of cholesterol from LDL seem to be involved in the greater accumulation of tissue cholesterol resulting from polyunsaturated fat feeding.
Lipids 20:757-764, 1985.

INTRODUCTION

Increased deposition of cholesterol in tissues after consumption of diets high in polyunsaturated fatty acids has been observed in a number of species (1-3). Total body cholesterol concentrations of swine were significantly increased when casein was fed, compared with soy protein (4). The mechanism for the accumulation of tissue cholesterol is unclear, but it is likely mediated by the interaction of LDL with the cells. LDL degradation was increased when linoleic acid was supplied to cultured fibroblasts in the medium (5). It has not been determined if a similar effect occurs in vivo. Changes in membrane lipid composition may have an effect on LDL receptor function or expression because the receptors are an integral part of the plasma membrane. Clandinin and colleagues have shown that the structural lipid composition of tissue plasma membranes can be altered in vivo by differences in dietary fat saturation (6). Changes in plasma membrane composition also may alter receptor-independent catabolism of LDL or the extent of cholesterol surface exchange. Transfer of unesterified cholesterol between serum lipoproteins and cell membranes can play an important role in regulation of cellular cholesterol because this process can result in the removal of cholesterol from cells by way of an acceptor, such as high density lipoprotein (HDL), as well as result in delivery of cholesterol to cells (7). Cholesterol exchange between serum lipoproteins is influenced primarily by lipid-lipid interactions and may lead to net transfer from LDL to the cells when the ratio of free cholesterol to phospholipid or cholesteryl esters in LDL has been changed (8). Entry of cholesterol by this route could be significant because, unlike the LDL-receptor pathway, this pathway is not regulated strictly. Changes in LDL composition induced by dietary fat or protein source also may alter LDL interactions with the receptor, and thus possibly influence tissue cholesterol concentrations through this route. The contribution of different tissues to the in vivo degradation of plasma LDL in pigs on a chow diet has been described (9), but very little information is available regarding the influence of diet on tissue contributions to LDL degradation.

Previous research in our laboratory suggested that dietary fat, and possibly dietary protein, may influence tissue degradation of in-

[1]Data taken from a dissertation submitted to Iowa State University by L. S. Walsh Hentges as partial fulfillment of the requirements for the Ph.D. degree. A preliminary paper was presented at the 68th Annual Meeting of the Federation of American Societies for Experimental Biology in St. Louis, Missouri, April 1984 (Fed. Proc. 43:796).

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tact LDL and surface exchange of free cholesterol from LDL to cells (10). The objective of this study was to determine the effect of dietary fat and protein source in a complex diet on the cholesterol concentrations of a variety of tissues, the uptake of intact LDL by those tissues, and the net exchange of free cholesterol from LDL to the tissues.

MATERIALS AND METHODS

Animals and Diets

Beginning at six wk of age, 24 miniature pigs were fed four isocaloric, isonitrogenous diets (six pigs/diet) in a 2 × 2 factorial arrangement. Diets contained raw egg white or soy protein isolate as the main protein source and rendered beef tallow or soybean oil as the main fat source. Details concerning the animals and diets have been published (11).

LDL Labeling and Uptake

After pigs were fed the diets for four wk, LDL were isolated by way of density adjustment and ultracentrifugation at 18 C (Model L-2 or L8-70 ultracentrifuge, Ti 50.3 rotor, Beckman Instruments Inc., Irvine, California) from plasma collected from each pig (12). The apoprotein component of the LDL then was labeled with [U-^{14}C]sucrose (555 mCi/mmol, Amersham, Arlington Heights, Illinois) by using the technique of Pittman et al. (9,13). This method produces LDL with the radioactive label covalently bound to the apolipoprotein. The lysosomal accumulation of [^{14}C]sucrose provided a cumulative tally of the amount of protein accumulated in a particular tissue. It has been estimated that 44–68% of the uptake of intact LDL is attributable to receptors (14). Additional plasma was used to label the unesterified cholesterol pool of LDL with [1-α,2-α-(n)-^{3}H]-cholesterol (47 Cu/mmol, Amersham, Arlington Heights, Illinois) by the technique of Schwartz et al. (15). After correction for the amount of cholesterol accounted for by uptake of intact LDL, the remainder of the [^{3}H]cholesterol that accumulated was assumed to have entered by exchange at the cell surface. This amount estimated the net transfer of cholesterol because [^{3}H]cholesterol may exchange back to LDL or into HDL.

The [^{3}H]cholesterol- and [^{14}C]sucrose-LDL from each pig were combined and autologous injections (average 1.9 μCi ^{3}H and 1.3 μCi ^{14}C/kg body weight) by way of the jugular vein were performed after the pigs had consumed the diets for six wk. Serial blood samples were collected for the next 48 hr; the specific radioactivity of [^{3}H]cholesterol- and [^{14}C]sucrose-LDL in the plasma was determined by using standard dual label scintillation counting techniques.

Tissue Collection and Analysis

Forty-eight hr after injection of labeled LDL, pigs were slaughtered by asphyxiation with carbon dioxide. Liver, other viscera, aorta, half the carcass (including head, skin and hair but not brain and spinal cord), and samples of adipose tissue (perirenal, subcutaneous and visceral) and muscle (M. sartorius and Latissimus dorsi) were collected from each pig. Tissues were trimmed of visible fat and connective tissue, rinsed, blotted dry and weighed. The carcass was weighed and frozen; a representative sample of the carcass then was ground (Universal Meat Chopper No. 304, Universal Chopper Div., Union Mfg. Co., Meriden, Connecticut). Carcass protein (N × 6.25) was determined by the Kjeldahl method (16). Carcass muscle was calculated from carcass protein by assuming muscle to be 30% protein and 70% water.

Portions of each sampled tissue were lyophilized, and the total lipid was extracted with chloroform/methanol (2:1, v/v) by the method of Rotenberg and Christensen (17). Total cholesterol concentrations were determined enzymatically (18) on aliquots of the total lipid extracts that were evaporated to dryness and redissolved in isopropanol. Total radioactivity of [^{3}H]cholesterol in the tissues was measured by scintillation counting of aliquots of the total lipid extracts that were evaporated to dryness and redissolved in scintillation cocktail. The external standard ratio method was used to correct for quenching.

To determine uptake of intact LDL, additional tissue samples were homogenized in five vol of water (Polytron homogenizer, Type PT10/35, Brinkman Instruments, Westbury, New York), twice frozen and thawed, and again homogenized. To adsorb undegraded LDL, 20 mg/ml of Cab-O-Sil (thixotropic gel powder, Packard Instrument Co. Inc., Downers Grove, Illinois) was added to each tissue homogenate (9). Samples were shaken vigorously for one hr with a wrist-action shaker and then centrifuged at 2000 × g for 30 min. Radioactivity of the supernatant fraction was determined by using standard scintillation counting techniques; counting efficiency was determined by use of an external standard.

Calculation of Uptake of LDL-Cholesterol

The relative contributions of the uptake of intact LDL and cell surface exchange to the ac-

cumulation of cellular cholesterol were calculated by using the following relationships:

(1) Amount of cholesterol entering by uptake of intact LDL =

$$^{14}\text{C dpm inside} \left(\frac{\text{mg LDL-protein}}{^{14}\text{C dpm}}\right)\left(\frac{\text{mg cholesterol}}{\text{mg LDL-protein}}\right)$$

(2) Net radioactivity entering by surface exchange =

$$^{3}\text{H dpm inside} - \left(\frac{\text{mg cholesterol entering}}{\text{by uptake of intact LDL}}\right)\frac{^{3}\text{H dpm}}{\text{mg LDL-chol}}$$

(3) Net amount of cholesterol entering by surface exchange =

$$\left(\frac{\text{Net } ^{3}\text{H dpm entering}}{\text{by surface exchange}}\right)\left(\frac{\text{mg LDL-chol}}{^{3}\text{H dpm}}\right)$$

Average specific radioactivity of [^{14}C]sucrose-LDL (mg LDL-protein/^{14}C dpm) and average specific radioactivity of [^{3}H]cholesterol-LDL (mg LDL-cholesterol/^{3}H dpm) in plasma during the 48 hr period were calculated from evenly-spaced points on the fitted curves for disappearance of radioactivity from the plasma.

Statistical Analysis

Data were subjected to the analysis of variance according to the factorial arrangement in a randomized block design by using the GLM program of the Statistical Analysis System (SAS) to determine the influence of fat and protein source and to test for interactions between fat and protein. Data are expressed as group means ± pooled standard errors of the mean.

RESULTS

The concentrations of cholesterol in several tissues are presented in Table 1. Aorta, heart, large intestine and small intestine had significantly greater ($P < .05$) concentrations of cholesterol, and liver, visceral adipose tissue, lungs and Latissimus dorsi muscle tended to have greater ($P > .05$) concentrations of cholesterol when soybean oil, compared with beef tallow, was fed. Dietary protein source had a significant effect ($P < .05$) on cholesterol concentrations in only three tissues; egg white consumption, in contrast to soy protein, increased

TABLE 1

Total Cholesterol Concentration in Tissues

Tissues	Diet[a]				Pooled SEM
	EW/SO	EW/BT	SP/SO	SP/BT	
	mg/g dry tissue				
Aorta	5.1[b]	4.5	4.9[b]	3.7	0.6
Adipose tissue					
Perirenal	5.6	5.7	5.5	6.3	0.6
Subcutaneous	4.1	4.5	3.9	3.9	0.6
Visceral	6.8	5.8	5.6	4.3	1.1
Urinary bladder	6.5	5.9	6.1	7.3	1.6
Carcass	7.5[c]	6.9[c]	4.5	5.0	1.1
Gall bladder	17.3[c]	16.2[c]	19.9	26.3	6.8
Heart	5.8[b]	4.8	5.5[b]	5.1	0.7
Kidney	11.6	10.2	11.9	11.3	1.5
Large intestine	12.9[b]	8.4	10.1[b]	7.8	1.3
Liver	9.9[d]	8.3	10.2[d]	9.4	0.6
Lungs	12.2	10.9	12.9	11.9	1.5
Skeletal muscle					
M. sartorius	4.0	3.9	4.3	4.3	0.5
Latissimus dorsi	4.9	3.7	4.6	4.3	0.9
Pancreas	9.2	7.3	7.8	8.4	1.0
Reproductive organs[e]	7.7	8.8	9.1	9.2	1.0
Small intestine	11.7[b]	9.2	11.6[b]	10.4	1.1
Spleen	10.3[c]	10.9[c]	13.5	13.2	2.1
Stomach	8.8	9.1	9.1	8.5	0.8

[a]EW = egg white, SP = soy protein isolate, SO = soybean oil, BT = beef tallow.

[b]Fat effect, $P < 0.05$.

[c]Protein effect, $P < 0.05$.

[d]Fat effect, $P < 0.10$.

[e]Testes were analyzed in male pigs; ovaries plus uterus were analyzed in female pigs.

TABLE 2

Uptake of LDL-Cholesterol in 48 Hr Accounted for by Uptake of Intact LDL

Tissue	Diet[a]				Pooled SEM
	EW/SO	EW/BT	SP/SO	SP/BT	
	mg/g wet tissue				
Aorta	.046	.014	.043	.031	.015
Adipose tissue					
Perirenal	.031	.015	.029	.028	.034
Subcutaneous	.009	.004	.009	.006	.003
Visceral	.011	.008	.021	.021	.006
Urinary bladder	.047	.033	.061	.089	.023
Carcass	.025	.010	.039	.037	.015
Gall bladder	.097	.071	.187	.193	.046
Heart	.016	.015	.032	.021	.008
Kidney	.463	.466	1.506	1.178	.388
Large intestine	.060	.038	.079	.139	.038
Liver	.594	.744	.856	1.230	.287
Lungs	.317	.155	.289	.354	.098
Skeletal muscle					
M. sartorius	.010	.005	.022	.012	.007
Latissimus dorsi	.012	.007	.007	.008	.003
Pancreas	.057	.025	.071	.047	.024
Reproductive organs[a]	.189	.124	.133	.217	.065
Small intestine	.054	.050	.080	.080	.013
Spleen	.184	.123	.210	.351	.049
Stomach	.018	.021	.042	.028	.013

[a]See Table 1.

cholesterol concentration in the carcass and decreased it in gall bladder and spleen. Cholesterol concentrations in urinary bladder, pancreas and stomach were not influenced by diet.

Tables 2 and 3 show the amount of LDL-cholesterol that accumulated in several tissues during the 48 hr period between injection of the labeled LDL and slaughter. Uptake of cholesterol by way of intact LDL (Table 2) was not significantly influenced by fat source, although aorta, subcutaneous adipose tissue, M. sartorius muscle and pancreas of pigs fed soybean oil tended to be greater ($P > .05$) compared with pigs fed beef tallow. Liver uptake of cholesterol by way of intact LDL tended to be less ($P > .05$) in pigs consuming soybean oil in contrast to beef tallow. No tissue was influenced significantly by dietary protein source, but uptake of cholesterol by way of intact LDL tended to be greater ($P > .05$) in most tissues studied when pigs were fed soy protein compared with egg white. Associations between uptake by way of intact LDL and tissue cholesterol concentrations were seen in only aorta and heart in response to dietary fat source and in only gall bladder, kidney, liver, M. sartorius muscle and spleen in response to dietary protein source. In these tissues, consumption of the vegetable product (soybean oil or soy protein) was associated with greater uptake and greater cholesterol concentrations than was consumption of the animal product.

Uptake of LDL-cholesterol by exchange of free cholesterol (Table 3) was not affected significantly by diet, except that uptake by exchange was greater ($P < .05$) in perirenal adipose tissue and reproductive organs of pigs fed soy protein than in those tissues of egg white-fed pigs. In general, uptake tended to be greater ($P > .05$) when the vegetable product was consumed. Aorta, gall bladder, liver, Latissimus dorsi and pancreas responded to fat source, whereas visceral adipose tissue, carcass and heart responded to protein source. Uptake of LDL-cholesterol by free cholesterol exchange was associated with tissue cholesterol concentration in only aorta and heart in response to fat source.

The relative contribution of several tissues to the total uptake of LDL-cholesterol is shown in Table 4. Data for the four diets have been pooled because diet had no significant effect on the relative activity of the tissues. Aortas of pigs fed soybean oil tended ($P < .06$) to be more active in the uptake of cholesterol by way of intact LDL. Liver was responsible for more than 50% of the uptake of cholesterol by way of intact LDL, with kidney, skeletal muscle and lungs combined contributing another 30%. Skeletal muscle and adipose tissue each were

TABLE 3

Uptake of LDL-Cholesterol in 48 Hr Accounted for by Net Free Cholesterol Exchange

Tissue	Diet[a]				Pooled SEM
	EW/SO	EW/BT	SP/SO	SP/BT	
	mg/g wet tissue				
Aorta	.044	.018	.031	.018	.014
Adipose tissue					
Perirenal	.070[b]	.049[b]	.087	.119	.025
Subcutaneous	.039	.025	.030	.101	.007
Visceral	.091	.053	.096	.102	.019
Urinary bladder	.010	.013	.014	.008	.008
Carcass	.061	.050	.066	.081	.013
Gall bladder	.976	.944	1.690	.849	.377
Heart	.082	.058	.087	.086	.024
Kidney	.0	.0	.0	.0	—
Large intestine	.0383	.112	.098	.154	.095
Liver	.098	.0	.056	.024	.038
Lungs	.214	.144	.207	.211	.093
Skeletal muscle					
M. sartorius	.028	.026	.026	.038	.009
Latissimus dorsi	.038	.023	.039	.030	.012
Pancreas	.243	.144	.229	.235	.102
Reproductive organs[a]	.013[b]	.045[b]	.239	.147	.046
Small intestine	.165	.103	.116	.194	.046
Spleen	.216	.253	.313	.150	.080
Stomach	.071	.051	.065	.047	.020

[a]See Table 1.

[b]Protein effect, $P < 0.05$.

TABLE 4

Tissue Contributions to LDL-Cholesterol Uptake in 48 Hr Accounted for by Uptake of Intact LDL and by Net Free Cholesterol Exchange

Tissue	Intact LDL (%)	Exchange (%)
Liver	55.12 ± 6.36[a]	3.17 ± 2.73
Kidney	12.55 ± 3.07	0.00 ± 0.00
Skeletal muscle	9.45 ± 2.99	31.16 ± 5.49
Lungs	9.26 ± 3.23	5.32 ± 1.79
Adipose tissue	4.98 ± 1.25	29.09 ± 5.01
Small intestine	3.91 ± 0.72	5.59 ± 0.82
Large intestine	2.75 ± 0.40	10.88 ± 5.01
Spleen	1.62 ± 0.55	1.78 ± 0.60
Reproductive organs[b]	1.00 ± 0.37	1.48 ± 1.08
Stomach	0.72 ± 0.21	2.30 ± 0.64
Heart	0.36 ± 0.05	1.78 ± 0.56
Pancreas	0.27 ± 0.10	2.36 ± 1.44
Urinary bladder	0.07 ± 0.02	0.02 ± 0.01
Gall bladder	0.04 ± 0.01	0.96 ± 0.43
Aorta	0.04 ± 0.01	0.07 ± 0.05
Soybean oil[c]	0.05 ± 0.02[d]	
Beef tallow[c]	0.03 ± 0.01[d]	

[a]Mean of all diets ± pooled SEM.

[b]See Table 1.

[c]Data from soybean oil-fed pigs and beef tallow-fed pigs are shown.

[d]Fat effect, $P = 0.06$.

responsible for ca. 30% of the uptake of cholesterol by way of exchange; large intestine and small intestine combined were responsible for about 15%.

DISCUSSION

As in other studies, soybean oil consumption resulted in greater cholesterol concentrations in several tissues. The most responsive tissues were those most strongly associated with cholesterol metabolism and atherosclerosis: aorta, heart, liver, small intestine and large intestine. Both skeletal muscle sites and the intact carcass also had greater cholesterol concentrations when soybean oil, in contrast to beef tallow, was fed, although not significantly so. Muscle has the potential to be a major storage organ for cholesterol because small differences in cholesterol concentration may represent large differences in the total amount of cholesterol accumulated in a tissue of this size. It is not known whether cholesterol stored in muscle (or other tissues) is easily mobilized at some later time or is relatively inert once deposited. This is a critical factor in determining whether the

effects of polyunsaturated fat feeding truly are antiatherogenic. The observation that the cholesterol concentration of aorta also increased with soybean oil feeding implies that increased tissue cholesterol accumulation is not totally benign.

The response of tissue cholesterol concentration to dietary protein source is much more variable than is the response to dietary fat source. In the present study, carcass had a significantly greater cholesterol concentration when egg white, rather than soy protein, was consumed, and aorta and subcutaneous and visceral adipose tissue showed similar trends. Egg white consumption, compared with soy protein, however, decreased cholesterol concentrations in a number of other tissues, including liver, M. sartorius muscle and kidney. Previous studies with a number of species have shown that casein, compared with soy protein, usually increases liver cholesterol but does not influence skin, muscle, adipose tissue or aorta cholesterol concentrations to a significant degree (4,19,23). Total body cholesterol concentration (without CNS) was increased when soy protein, compared with casein, was fed to pigs (4), but was decreased when a beef-based, rather than soy-based, diet was fed (10). Other sources of animal protein had variable effects on liver and aorta cholesterol concentrations compared with soy protein (23–25). Thus, as our results have indicated, the effect of protein on tissue cholesterol concentrations is not firmly established. The amino acid composition of a protein, particularly the ratio of arginine and lysine, has been proposed as a possible determinant of the dietary protein effect on plasma cholesterol concentrations (26) and also may influence tissue cholesterol concentrations, but a detailed mechanism for either effect remains to be elucidated.

Uptake of cholesterol by degradation of intact LDL was influenced to some extent by dietary fat and protein sources. Both soybean oil and soy protein feeding tended to result in greater catabolism of LDL by this route than did beef tallow or egg white consumption. Data from this study cannot be used to determine the relative contributions of receptor-dependent and receptor-independent mechanisms to the uptake of intact LDL, but other studies have shown that receptor-dependent catabolism accounts for ca. 65% of hepatic LDL degradation and about 60% overall in animals fed chow diets (14,27). The available evidence suggests that receptor-dependent degradation may be sensitive to changes in the degree of unsaturation of the dietary lipid (5) and to changes in protein source (28), but the susceptibility of receptor-independent catabolism of LDL to dietary manipulation is unknown.

Efrati et al. (29) demonstrated that both free and esterified cholesterol can be transferred from LDL to mycoplasma membranes by a simple exchange process that does not involve prolonged contact or fusion of the particle with the membrane nor degradation of the particle. Stender and Zilversmit (30) studied this uptake mechanism in vivo and found it to account for as much as 80% of the total free cholesterol influx in some tissues. In the present study, the net exchange of unesterified cholesterol from LDL to the tissues did not seem to be a significant factor in accounting for the accumulation of cholesterol in the tissues of soybean oil-fed pigs. However, the period over which uptake was measured (48 hr) may have been too short to allow for the expression of subtle, but potentially significant, differences in uptake by this mechanism. This may be particularly important in the aorta because the pathological accumulation of cholesterol would be uncontrolled if cholesterol entered the arterial wall by a route other than the tightly regulated LDL receptor mechanism, and atherosclerosis typically takes years to develop.

It is intriguing that associations between uptake by either mechanism and tissue cholesterol concentrations were seen in only a few tissues. This suggests that dietary factors that influence tissue cholesterol accumulation also must influence other points of cholesterol metabolism, such as cholesterol synthesis and excretion or absorption of cholesterol from the gut.

The relative contribution of the several tissues to the uptake of intact LDL was similar to that previously reported for swine (9,31). The seemingly large contribution of the kidney can be explained in that the small amount of the [^{14}C]sucrose degradation products that escapes the tissues is excreted in the urine and thus would be expected to accumulate in the kidney until excreted. Aorta, although contributing only a small proportion to the total amount of uptake of intact LDL, was the only tissue that showed a responsiveness to dietary treatment. This observation, along with the fact that aorta was one of the few tissues in which uptake of intact LDL was associated with the tissue cholesterol concentration, suggests that aorta, and possibly the arteries in general, may be very different than other tissues, in that uptake of LDL may be an important mechanism by which dietary factors influence the total tissue cholesterol concentration. This difference between aorta and other tissues may be critical in the etiology of atherosclerosis.

The largest proportion of the total net exchange of free cholesterol predictably occurred in those tissues that compose the greatest proportion of the whole body: skeletal muscle and adipose tissue. Large and small intestine also contributed significantly to free cholesterol exchange. This might be expected because the epithelial cells of the gastrointestinal tract turn over rapidly and thus would be more likely to take up cholesterol by this mechanism based solely on their accelerated mitotic and metabolic rates.

All the tissues studied contributed to LDL-cholesterol uptake. Differences in the relative contributions of the tissues to the two entry mechanisms measured suggest that the several tissues play different roles in the maintenance of balance in whole body cholesterol metabolism.

We had expected that animals whose tissues had greater concentrations of cholesterol because of diet composition would have LDL with a different composition and concentration and a more rapid clearance rate from plasma, and would have a greater accumulation of LDL-cholesterol in those tissues. We found that beef tallow, in contrast to soybean oil, increased plasma cholesterol concentrations and the ratio of HDL- to LDL-cholesterol and decreased the weight percentage of cholesterol in LDL. It had no effect on clearance of LDL from plasma (10), while decreasing the cholesterol concentration in tissues and having a variable effect on uptake of LDL by tissues. Protein source did not have a significant effect on plasma cholesterol concentration, distribution of lipoprotein cholesterol or LDL composition. However, egg white consumption, compared with soy protein, resulted in slower clearance of LDL from the circulation (10), while having no significant influence on tissue cholesterol concentrations nor on uptake of LDL-cholesterol in most tissues. Thus, dietary fat and protein seemed to exert their effects on cholesterol metabolism through different mechanisms, and mechanisms other than uptake of LDL-cholesterol may be responsible for the greater accumulation of tissue cholesterol that resulted from soybean oil feeding. It has been shown that cholesterol biosynthesis (32-34), absorption (32,34,35) and excretion (19,36-38) may be influenced by dietary fat and/or protein source. Additional research needs to be done to determine the extent of these influences and the extent to which these processes contribute to the accumulation of cholesterol in the tissues.

We have found that dietary protein and fat sources act independently to influence tissue cholesterol concentrations as well as cholesterol transport and clearance (11). Uptake of LDL-cholesterol was not necessarily related to tissue cholesterol concentrations and varied between tissues. There were only a few tissues in which cholesterol concentration was associated with uptake of LDL-cholesterol; in these tissues (including aorta), consumption of soybean oil was associated with greater uptake and cholesterol concentrations than consumption of beef tallow. The data suggest that different mechanisms mediate the influences of dietary protein and fat on cholesterol metabolism, and that cholesterol biosynthesis, absorption, and/or excretion may be more important than uptake of LDL-cholesterol in the maintenance of cholesterol homeostasis in the whole body.

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Hydrolysis of Intralipid by Pancreatic Lipase and Phospholipase A_2-Gel Filtration Studies

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ABSTRACT

Intralipid was incubated with pancreatic lipase (EC 3.1.1.3) and/or phospholipase A_2 (EC 3.1.1.4) at two bile salts/phosphatidylcholine molar ratios and at two different triglyceride hydrolysis rates using various amounts of lipase.

Incubations were studied by gel filtration. Results show: (i) During lipase action, three phases of lipids coexist: an emulsified phase, a micellar phase and an intermediate heavy phase sized between the two others. The equilibrium between each phase is dependent upon the bile salts concentration. (ii) Under these conditions, pancreatic lipase was at 60% bound to the emulsified phase, whereas pancreatic phospholipase A_2 was bound at 94% to the micellar phase.
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INTRODUCTION

After their ingestion, dietary triglycerides are hydrolyzed in the stomach by lingual lipase, which liberates diglycerides and fatty acids (1,2). These products, together with phospholipids, promote emulsification of triglycerides in the stomach, leading to phospholipid-coated triglyceride particles. These particles are then propelled through the pylorus into the duodenum where they are subjected to the detergent effect of bile salts.

Using phosphatidylcholine-coated triglyceride particles (3-6) or egg yolk emulsion (6), several authors have shown that bile salts dissociate a considerable fraction of phosphatidylcholine initially associated with neutral lipids, leading to the coexistence of an emulsified phase and a phase of mixed phosphatidylcholine-bile salt micelles (3,6).

If, logically, the emulsified phase constitutes the only hydrolyzable substrate for pancreatic lipase, nevertheless two kinds of substrates are offered to the pancreatic phospholipase A_2. Using different types of substrates (3,6), we have clearly shown that pancreatic phospholipase A_2 is almost entirely bound to the micellar phase, suggesting that this enzyme acts only on phospholipids organized in micellar structures.

On the other hand, a synergistic effect of pancreatic phospholipase A_2 on lipase action has been described in vitro (4,5,7). It was observed that the binding of lipase on phosphatidylcholine-covered triglyceride droplets was enhanced by the action of phospholipase A_2. Two mechanisms can be advanced to explain this synergistic effect. First, the hydrolysis products generated by phospholipase A_2 at the droplets interface play a role in the binding of lipase to the particles (4,7). However, the fact that we found only a small percentage of phospholipase A_2 (5%) bound to the emulsified phase (3,6) makes this hypothesis questionable. Second, it has been shown (5,8,9) that lipase can be partitioned between a triglyceride-water interface and mixed micelles of phosphatidylcholine and bile salts which represent another type of lipid-water interface. When pancreatic phospholipase A_2 hydrolyzes these micelles, lipase could be displaced toward the emulsified phase, resulting in a diminution of a lag phase.

The major consequence of the pancreatic lipase action on triglycerides is the appearance of diglycerides, fatty acids and monoglycerides. Since the studies of Hofmann and Borgstrom (10), it has been well established that lipolysis products (i.e. fatty acids and monoglycerides) are solubilized together with phospholipids and cholesterol by lysophospholipids and by bile salts in a unique micellar phase. This phase is isolated either by high speed centrifugation (10) or by gel filtration of the crude duodenal content (11) or by multiple ultrafiltrations of the aqueous phase isolated after mild centrifugation (12).

However, on the basis of recent data, some authors have introduced the idea of intermediate structures between the emulsified phase and the micellar phase. During triglyceride hydrolysis, these authors showed by light microscopy (13) or by freeze-fracture techniques (14) the appearance of a liquid crystalline phase close to the globule interface. These results,

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taken together with others (15), led to the conclusion that the aqueous phase was composed of at least two dispersed phases: a phase of large micelles composed of fatty acids, monoglycerides, phosphatidylcholines and traces of diglycerides, and a liquid crystalline phase containing the same compounds; the transformation from one to the other would be a function of their saturation with bile salts (16).

Inasmuch as previous data showing the synergistic effect of phospholipase A_2 on lipase action are for the most part bulk kinetic studies, the purpose of our paper is to bring information on how lipase and phospholipase behave when they are mixed together with a phospholipid-coated triglyceride emulsion in the presence of bile salts. At the same time, the fate of the emulsion under the action of these enzymes was investigated.

Results obtained from gel filtration studies suggest that each enzyme binds to its substrate and acts independently of the other. Lipase induces the appearance of a lipidic phase sized between the emulsified phase and the micellar phase. Results also emphasize the role of bile salts in the solubilization of lipolytic products through these intermediate and micellar phases.

MATERIALS AND METHODS

Preparation of Substrates

Ten percent Intralipid® emulsion was obtained from KabiVitrum (Paris). Intralipid was centrifuged with a 40.3 rotor in a Beckman L5-75B ultracentrifuge as previously described (3) and reconstituted with standard buffer (0.01 M tris-HCl pH 7.0; 0.1 M NaCl; 1 mM $CaCl_2$; 0.02% NaN_2) in order to obtain a 20% emulsion. It was stored at 4 C in a nitrogen medium and used within three to four days. In order to quantitate the extent of hydrolysis rate and the chromatographic behavior of lipolytic products, the emulsion was labelled with [1-^{14}C] triolein (CEA, France—sp. act. 52.0 mCi/mmol). Three ml of this emulsion (660 μmol of triglycerides, 42 μmol of phosphatidylcholines) were incubated overnight under nitrogen and gentle stirring with 0.73 μCi of [1-^{14}C] triolein previously evaporated to dryness. We checked that the incorporation of [1-^{14}C] triolein was complete by measuring simultaneously the release of fatty acids following two methods: one by measuring potentiometrically the liberation of fatty acids (see below enzyme assays), the second one by measuring the amount of liberated [1-^{14}C] oleic acid after extraction of lipids according to the Folch method. If incorporation was complete, the comparison of the two methods should give approximately the same specific activity for lipase.

Enzymes

Porcine pancreatic lipase and colipase were purchased from Boehringer Mannheim and were freed of phospholipase A_2 activity using the standard egg yolk emulsion (17). Lipase activity was measured potentiometrically with a pH Stat (Radiometer TTT2, autoburette BU12) at pH 7.5 and at 25 C using tributyrin saturated with colipase as previously described (9). The lipase activity was expressed as enzyme units. One unit corresponds to the liberation of one microequivalent of fatty acid per min. Colipase saturation in the fractions was determined by measuring the difference of lipase activity between saturating colipase conditions and without colipase addition. Tritiated amidinated porcine pancreatic phospholipase A_2 (sp. radioactivity: 5.9 μCi/mg) was a gift of Dr. De Haas (Utrecht, Holland).

Incubation of Labelled Intralipid with Bile Salts and Pancreatic Enzymes

Labelled intralipid emulsion was incubated with variable quantities of the bile salts mixture (see below) in solution at pH 7.0 in such a way as to obtain the bile salts/phosphatidylcholine molar ratios desired. The phosphatidylcholine concentrations were obtained by diluting the incubation mixture with standard buffer. The incubation mixture (final volume of 2 ml) at room temperature was stirred gently for 30 min. An aliquot of 1.5 ml was then removed for gel filtration.

In the experiments with lipase and/or phospholipase A_2, enzymes were added after the 30 min of stirring and incubated for 1 min at 4 C. An aliquot of 1.5 ml was deposited immediately on the gel. Due to the viscosity of intralipid, the loading of the sample lasted about 10 min. Details of each experiment are given in the legend to the corresponding figure.

Gel Filtrations

All gel filtrations were performed in an 80 × 1.6 cm column filled with agarose A_4 (I.B.F., France) and equilibrated with standard buffer containing a mixture of four bile salts (molar percentage: glycocholate 29%, glycodeoxycholate 40%, taurocholate 8%, taurodeoxycholate 23%) at a total concentration of 1.2 mM. All bile salts came from Calbiochem (A grade).

All gel filtrations were done at 4 C, at a flow rate of 12–15 ml/hr. Fractions of 2.2 ml were

collected. After five or six gel filtrations, the gel was discarded and the column filled with a new gel.

Calibration of the column was done with standard proteins: blue dextran (void volume), thyroglobuline (Mr: 669 KDa), ferritine (Mr: 440 KDa), catalase (Mr: 240 KDa), serum albumin (Mr: 69 KDa), vitamin B_{12} (total volume).

Assays

Lipidic phosphorus was assayed semi-automatically (18). Bile salts were assayed enzymatically by an automatic method (19). Identification and quantification of [1-^{14}C] triolein, [1-^{14}C] diolein, [1-^{14}C] monoolein and [1-^{14}C] fatty acids were done after a Folch extraction (20) of the pooled fractions corresponding to each peak and separation performed on thin layer chromatography (TLC). The Schleicher and Schull F 1500 plates were developed using heptane/diethylether/formic acid (90:60:4, v/v/v) as mobile phase (21). The spots were located by iodine vapors, scraped off and counted in a liquid scintillation spectrophotometer (Beckman LS 9000) in 10 ml of Readysolve MP (Beckman) scintillation fluid. In some experiments with pancreatic phospholipase, the phosphatidylcholine and lysophosphatidylcholine were separated by TLC and assayed as previously described (11).

RESULTS

The control of the incorporation of [1-^{14}C] triolein was done by two assay methods as described in Materials and Methods. By the quantification of the radioactive liberated fatty acids, we determined a lipase activity which is about 80% of that measured with the bulk potentiometric assay. We can reasonably conclude that the incorporation of [1-^{14}C] triolein into the emulsion has been complete.

Labelled intralipid was chromatographed alone on agarose A_4 in bile salts-containing buffer at a bile salts concentration of 1.2 mM. The total triglycerides and phosphatidylcholines were eluted at the void volume, which indicates that the emulsion remained stable during the gel filtration.

When labelled intralipid was incubated with bile salts at a bile salts/phosphatidylcholine molar ratio of 4 and then filtered under the same conditions (Fig. 1), we observed two peaks of phospholipids. One is eluted at the void volume in lactescent fractions and contains 41% of phosphatidylcholines, and all the recovered triglycerides. The second one eluted at Kav = 0.74 in limpid fractions contains 51% of phosphatidylcholines, and is associated with a peak of bile salts. Comparable profiles were obtained when intralipid was incubated at a bile salts/phosphatidylcholine molar ratio of 2.

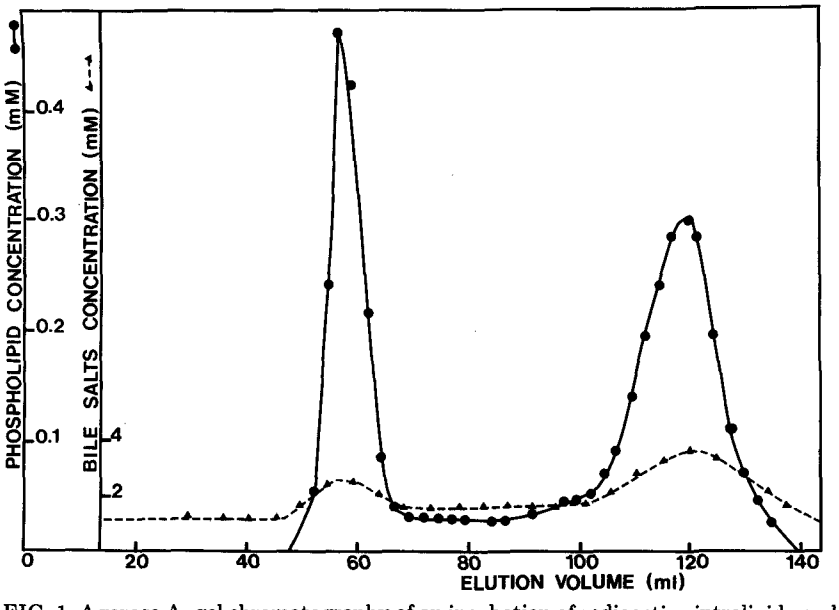

FIG. 1. Agarose A_4 gel chromatography of an incubation of radioactive intralipid emulsion in the presence of bile salts (bile salts/phosphatidylcholines = 4). The 2-ml incubation mixture contained 14 μmol of phosphatidylcholines, 220 μmol of triglycerides, 56 μmol of bile salts. 1.5 ml was chromatographed.

However, the amount of lipids eluted at a Kav of 0.74 is slightly lower than that obtained in Figure 1, consistent with previous data (6). Regardless of the bile salts/phosphatidylcholine molar ratio, the amount of phosphatidylcholines eluted between the two peaks represents about 8% of the eluted phosphatidylcholines. In the above experiments, the phospholipids recovery is ca. 87%, while that of triglycerides is 85%.

Gel Filtration of Intralipid

During a first series of experiments, we carried out incubations at a bile salts/phosphatidylcholine molar ratio of 4. Colipase and 120 units of lipase (measured on tributyrin) were added and incubated as described in Materials and Methods. Gel filtration was performed, and showed (Fig. 2) that the elution profile of phospholipids was markedly modified compared to Figure 1.

We can observe three peaks of lipids in which the respective lipid composition is given in Table 1. The first one was eluted at the void volume in lactescent fractions, while the peak eluted with a Kav of 0.74 was associated with a peak of bile salts. When compared to the preceding system, an intermediate peak of lipids appeared in slightly opalescent fractions with a Kav centered at about 0.32. A slight increase in bile salts above the baseline of 1.2 mM could be detected under this peak. The analysis of neutral lipids listed in Table 1 shows that:

(i) A hydrolysis rate of 50% was found. This rate was calculated on the basis of the amount of triglycerides recovered after the gel filtration of the incubation without the addition of lipase. This percentage represents the hydrolysis which occurred during incubation, loading, gel filtration until the extraction of lipids.

(ii) Triglycerides are essentially eluted with the excluded peak, together with diglycerides, monoglycerides and fatty acids.

(iii) Monoglycerides, diglycerides and fatty acids are present with the intermediate peak.

(iv) With the peak eluted with a Kav of 0.74, only fatty acids and monoglycerides are present.

The elution profile of lipase is shown in Figure 2. The percentage of lipase excluded with the emulsified phase is ca. 60%, and the enzyme is saturated with colipase. The rest is eluted in retarded fractions. This unbound lipase emulsion could represent the ability of the enzyme to bind to lipidic structures other than the lipidic globule (5,8,9).

At a bile salts/phosphatidylcholine molar ratio of 2 and with the same amount of lipase, a chromatographic distribution of phospholipids comparable to that in Figure 2 was obtained (data not shown). However, the inter-

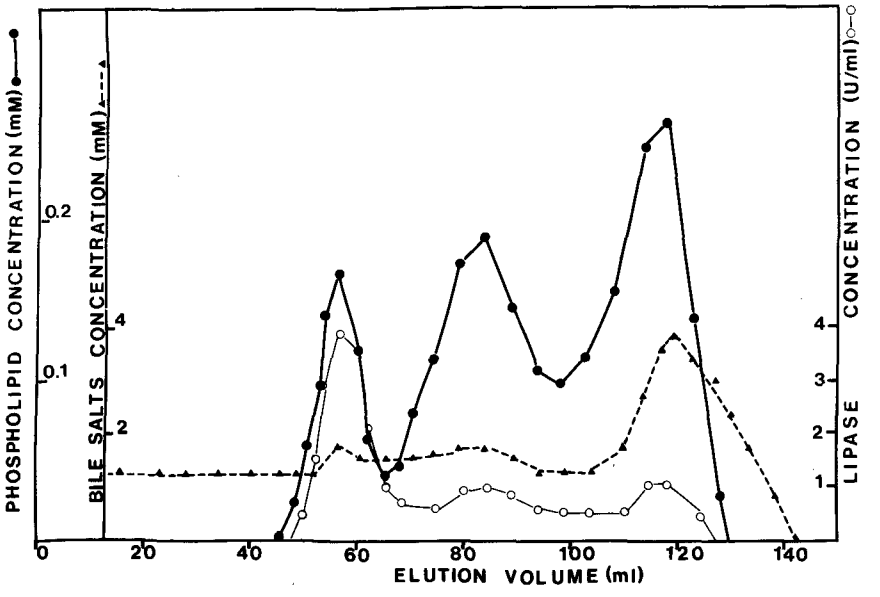

FIG. 2. Agarose A_4 gel chromatography of an incubation of radioactive intralipid emulsion in the presence of bile salts (bile salts/phosphatidylcholines = 4) colipase and lipase. The 2-ml incubation mixture contained 14 μmol of phosphatidylcholines, 220 μmol of triglycerides, 56 μmol of bile salts, 120 lipase units and colipase in saturating conditions. 1.5 ml was chromatographed.

TABLE 1

Distribution of Lipids after Gel Filtration of [1-^{14}C] Triolein Intralipid With or Without Lipase

	Radioactive intralipid no lipase added (Fig. 1)		Radioactive intralipid incubated with lipase Hydrolysis 50% (Fig. 2)			Radioactive intralipid incubated with lipase Hydrolysis 68% (Fig. 3)	
μmol	Peak I	Peak III	Peak I	Peak II	Peak III	Peak I	Peak II
Class I							
Triglycerides	140.0	0	71.6	0	0	46.0	0
Diglycerides	0	0	12.6	5.2	0	5.0	6.5
Class II							
Monoglycerides	0	0	21.5	16.0	29.0	43.0	47.5
Phospholipids	3.7	4.6	1.3	2.5	3.5	3.5	4.1
Class III							
Fatty acids	0	0	81.3	33.8	39.3	94.6	96.2
Bile salts	46.6	131.0	30.0	46.0	120.0	43.8	83.8
Class III/Class II and/or Class I	0.3	28.4	1.0	3.3	4.9	1.4	3.0

All the samples have been pre-incubated with bile salts at a BS/PC = 4.

Peak I refers to the voided emulsified phase, peak II to the intermediate phase and peak III to the micellar phase. Classes I, II and III refer to the classification of lipids (22,23).

mediate peak is generally less sharp than that obtained in Figure 2. Basically, we obtained a better resolution of the intermediate peak at a bile salts/phosphatidylcholine molar ratio of 4 than of 2.

During a second series of experiments, we carried out incubations with a greater amount of lipase units (240 units measured on tributyrin).

Figure 3 shows the elution pattern of such an incubation at a bile salts/phosphatidylcholine molar ratio of 4. The elution profile of lipids is dramatically modified because the peak initially eluted with a Kav:0.74 has now disappeared. About 40% of phosphatidylcholines were eluted with the void volume in turbid fractions followed by a shoulder containing ca. 50% of phosphatidylcholines. The respective lipid composition is given in Table 1. Compared to the above results (Fig. 2), the milky-like appearance of the excluded fractions disappeared, indicating that lipolysis was more pronounced; this is consistent with the 68% of hydrolysis rate found. Most of the lipase and its co-factor were excluded and represent about 75% of the recovered enzyme.

Incubations performed under the same experimental conditions but at a bile salts/phosphatidylcholine molar ratio of 2 gave a more markedly modified elution pattern of lipids (data not shown). In this case, we have only a sharp peak of lipids at the exclusion volume. It contains the total phosphatidylcholines, fatty acids, triglycerides, diglycerides and monoglycerides and 77% of the recovered lipase and colipase.

In all the experiments described above, recoveries of phospholipids and enzyme were found at least equal to 70% and 85%, respectively. Recovery of neutral lipids was at least 89%.

Under our present conditions, we carried out experiments in order to study the complete system.

First, when [^{3}H] amidinated phospholipase A_2 was incubated alone with intralipid at a bile salts/phosphatidylcholine ratio of 4, recovery was 96% with the peak eluted at a Kav:0.74 (insert Fig. 4), as it has been described previously (6). The distribution of phosphatidylcholines is quite similar to that obtained in the absence of phospholipase. Contrary to the lipase effect (Fig. 2), we at no time observed the appearance of an intermediate peak of phospholipids regardless of the amount of phospholipase added. Then we carried out incubations in which lipase, colipase and phospholipase were added in a proportion that reproduces their respective activities in vivo (16) (Fig. 4). The chromatographic behavior of lipidic phosphorus and neutral lipids is comparable to that obtained without phospholipase (Fig. 2). In the last peak, lysophosphatidylcholines produced by phospholipase represent about 47% of the lipidic phosphorus which represents 1.6 μmoles of lysophosphatidylcholines. The [^{3}H] amidinated phospholipase was at 94% recovered with the peak (Kav = 0.74) whereas 5% was excluded.

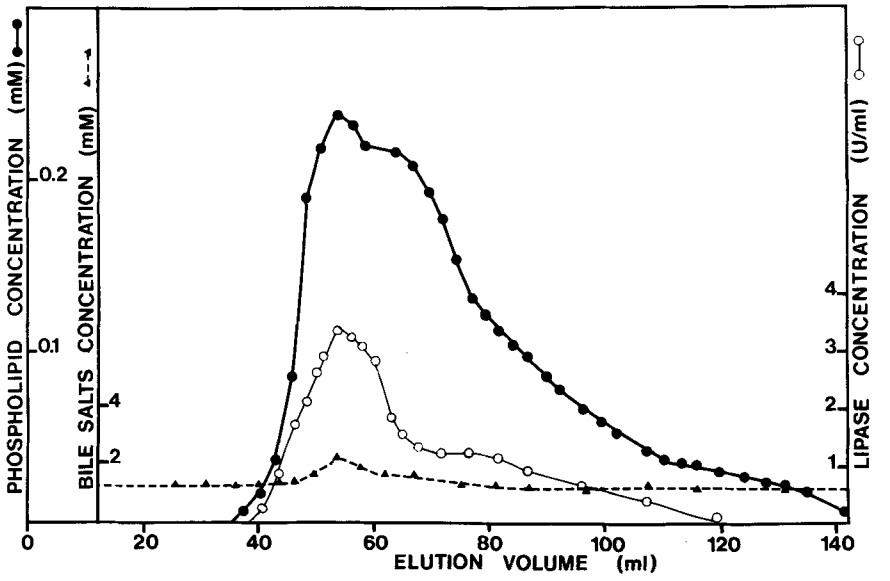

FIG. 3. Agarose A_4 gel chromatography of an incubation of radioactive intralipid emulsion in the presence of bile salts (bile salts/phosphatidylcholines = 4) colipase and lipase. The 2-ml incubation mixture contained 14 μmoles of phosphatidylcholines, 220 μmol of triglycerides, 56 μmoles of bile salts, 240 lipase units and colipase in saturating conditions. 1.5 ml was chromatographed.

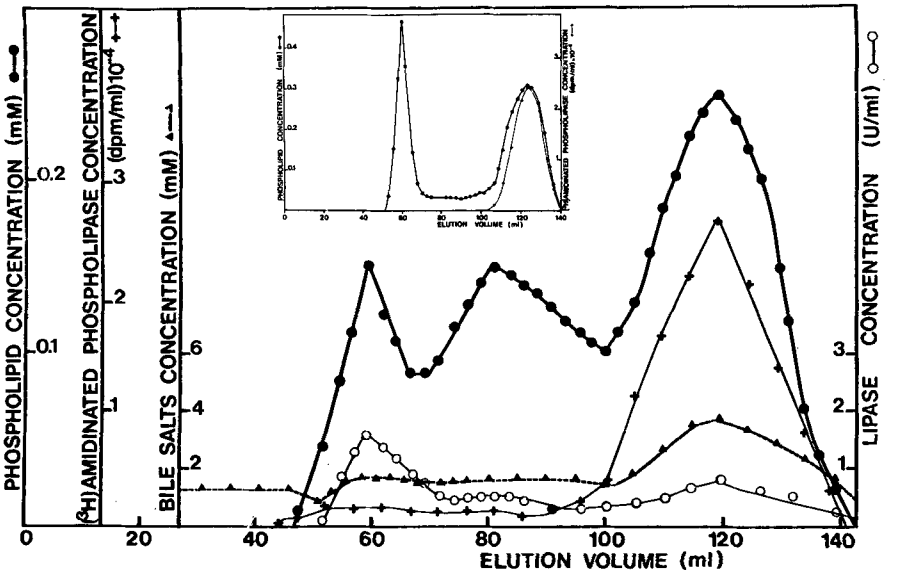

FIG. 4. Agarose A_4 gel chromatography of an incubation of radioactive intralipid emulsion in the presence of bile salts (bile salts/phosphatidylcholines = 4) colipase, lipase and [^{3}H] amidinated phospholipase. The 2-ml incubation mixture contained 14 μmoles of phosphatidylcholines, 220 μmol of triglycerides, 56 μmoles of bile salts, 120 lipase units with colipase in saturating conditions and 3 phospholipase A_2 units. 1.5 ml was chromatographed. Insert: Agarose A_4 gel chromatography of an incubation of intralipid emulsion in the presence of bile salts (bile salts/phosphatidylcholines = 4) and [^{3}H] amidinated phospholipase. The 2-ml incubation mixture contained 14 μmoles of phosphatidylcholines, 220 μmol of triglycerides, 56 μmoles of bile salts and 3 phospholipase A_2 units. 1.5 ml was chromatographed.

Recoveries of phospholipids and phospholipase were 70% and 90%, respectively. However, compared to the above gel filtrations in which lipase was 85% recovered (Figs. 2 and 3), the recoveries of lipase in these experiments did not exceed 55%. This unexplained loss of lipase units during gel filtration essentially was restricted to the lipase normally eluted with the emulsified phase. Indeed, the amount of lipase eluted in the retarded fractions is about the same as that found in the previous filtrations (Fig. 2). Under these conditions, only 36% of the recovered lipase was found with the emulsified phase. Taking into account the loss of lipase in this phase, and on the basis of the usual percentage of recovery obtained (85%), the fraction of lipase which should be bound to the emulsion is around 57%. This percentage is close to that obtained in Figure 2, 60%.

DISCUSSION

The present research was undertaken in an attempt to study the behavior of phospho-(lipases) in the presence of phospholipid-coated triglyceride particles such as that encountered during fat digestion.

The influence of these enzymes on the physicochemical state of this substrate under different conditions of hydrolysis was investigated. In order to estimate the in vivo situation, we tried to dissociate the different steps occurring during fat digestion.

First, it is known that the emulsion expelled from the stomach is composed essentially of triglycerides, diglycerides, fatty acids and phospholipids (16). Although intralipid does not perfectly imitate such an emulsion, it represents a good model of phospholipid-coated triglyceride globules. The incubation of such an emulsion with bile salts resulted in the formation of bile salts/phosphatidylcholine mixed micelles with an apparent Mr of 10^2 KDa (Fig. 1), which is in accordance with previous data (4-6). The emulsified phase that coexists with this micellar phase represents the available substrate for lipase. As shown in Figures 2, 3 and 4, a majority of lipase and colipase is bound to the emulsified phase.

However, the lipase induces a marked modification of the elution profile of lipids. This suggests a physicochemical reorganization or transformation of the emulsion induced by the hydrolytic action of the enzyme. Essentially these modifications are a function of two factors: the bile salts concentration and the extent of triglyceride hydrolysis.

At about 50% hydrolysis rate, a new phase of lipids appears. It is sized between the emulsified phase and micellar phase and, on the basis of protein calibration, its apparent molecular weight is estimated at 2×10^3 Kda. This phase contains phosphatidylcholines, lipolytic products (diglycerides, monoglycerides, fatty acids) and bile salts. On the basis of lipids classification (22,23), we can determine the ratios of the lipids of class III (fatty acids, bile salts) over the lipids of class II and/or I (phospholipids, monoglycerides, diglycerides, triglycerides). These ratios are 1, 3.3 and 5 in the voided, intermediate and micellar phase, respectively. These values and the apparent Mr determination of the corresponding structures allow us to propose three different types of lipids organization. The peak I is consistent with an emulsified phase. The ratio of 5 is compatible with a phase of light mixed micellar structures, while the ratio of 3.3 could correspond to a phase of very large micelles or liquid crystalline liposomal phase saturated with bile salts as already mentioned (16). The equilibrium between large micellar structures to light micellar structures is dependent upon the bile salts concentration. Higher incorporation of bile salts in the large structures should result in the appearance of light micellar structures (16). This phenomenon may occur naturally during gel filtration if the bile salts concentration of the eluent is much too high (11,24,25). The intermicellar bile salts concentration and the nature of bile salts are important in this process (24,26,27).

At about 70% triglyceride hydrolysis rate, the striking fact is the disappearance of the micellar phase (cf. Fig. 3). This is the result of a displacement of the equilibrium of bile salts, inducing aggregation of the light micelles into larger structures. The class III over class I and/or class II ratio in the intermediate phase is 3, which is comparable to that obtained with the 50% hydrolysis rate. This phenomenon is more pronounced when the bile salts concentration is reduced, because in this case we observe that both micellar and intermediate heavy phases disappear, their components being co-excluded with the emulsified phase.

Compared to lipase, the phospholipase effect is simpler. Phospholipase did not induce any significant change in the elution pattern of lipids (Fig. 4). The enzyme is, in its totality, bound to the light micellar phase (3,6). As we discussed previously (6), this raises the question of how the synergistic effect of phospholipase on lipase action may occur (4), inasmuch as phospholipase is found in a small percentage on the emulsified phase.

Although in some conditions (4) the in vitro synergistic effect of pure phospholipase A_2 is

undeniable, it seems that in vivo other factors like calcium and bile salts (4) or fatty acids generated for example by lingual lipase (28) are much more important in the binding process of lipase and colipase to the substrate.

In conclusion, from our results and others (13,16), it appears now clearly established that a large intermediate structure containing lipolytic products appears in the aqueous medium during lipolysis. This poses the question of the in vivo role of this phase in the process of lipid absorption.

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The Relative Incorporation of Arachidonic and Eicosapentaenoic Acids into Human Platelet Phospholipids

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ABSTRACT

The incorporation of arachidonic acid (AA) as compared to eicosapentaenoic acid (EPA) into human platelet phospholipids was tested by incubating washed platelets with a known mixture of [^{3}H]AA and [^{14}C]EPA. Following incubation, the platelet lipids were extracted, the individual phospholipids—phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylethanolamine (PE)— were separated by thin layer chromatography, and their corresponding [^{3}H]/[^{14}C] ratios were determined. Based on a [^{3}H]/[^{14}C] ratio of unity for the substrate mixture, the PC, PS, PI and PE exhibited ratios of 0.55, 0.93, 1.12 and 0.74, respectively, which were significantly different from 1.00 in all instances except in the case of PS. These results indicate that PC and PE selectively incorporated EPA, while PI showed preference toward AA. These selectivities may account partly for the differing AA/EPA mass ratios that have been observed among the individual phospholipids of human subjects consuming fish oils.

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INTRODUCTION

It has been documented recently that human subjects consuming fish or fish oils containing EPA exhibit prolonged bleeding times and diminished platelet reactivity to agonists (1-13). This decreased platelet reactivity suggests that dietary EPA may be protective against arterial thrombosis and atherosclerotic cardiovascular disease (2). The consumption of fish oils containing EPA also results in an altered fatty acid composition of the platelet phospholipids such that the percentage of EPA is increased while that of AA is decreased (3-10). Differences in the percentage of EPA associated with each of the individual phospholipids have been reported, as have the AA/EPA mass ratios (8,11). For example, AA/EPA ratios calculated for individual platelet phospholipids from the data of Brox et al. (11) were 4.7, 6.2 and 56.8 for PC, PE and PS+PI, respectively; Ahmed and Holub (8) reported mass ratios of 3.3, 7.1, 22.7 and 56.6 for PC, PE, PS and PI, respectively, following the consumption of cod liver oil. Differences in the mass ratios may reflect differences in incorporation and/or release of AA versus EPA, as well as the AA- and EPA-containing phospholipid acquired during platelet formation via the megakaryocyte (14) or transfer from serum lipoproteins (15,16). Recently, we have shown that EPA can inhibit the incorporation of AA into the phospholipids of washed human platelets in suspension (17). Although some differences in inhibition were observed across the various individual phospholipids, these differences were not sufficient to explain the variation in AA/EPA mass ratios observed among the individual platelet phospholipids of human subjects after fish oil consumption. This work was undertaken, therefore, to test for any differential incorporation of EPA as compared to AA into individual phospholipids of human platelets when these two polyunsaturated fatty acids were presented as dual-labelled mixtures.

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MATERIALS AND METHODS

The [1-^{14}C]eicosapentaenoic acid (56-57 mCi/mmol) and the [5,6,8,9,11,12,14,15-^{3}H(N)]-arachidonic acid (84-87 Ci/mmol) were obtained from New England Nuclear Corp. (Boston, Massachusetts) with radiochemical purities greater than 97% and 98%, respectively. The arachidonic acid, with a purity of 99%, was purchased from Nu Chek Prep (Elysian, Minnesota). Merck silica gel 60 HR (extra pure) was obtained from Terochem Laboratories Ltd. (Toronto, Ontario). Blood collection bags were obtained from Travenol Canada Inc., Mississauga, Ontario. All chemicals and solvents employed were of analytical grade. Siliconized glassware was used during the preparation and incubation of platelet suspensions.

Mixtures of [^{14}C]eicosapentaenoic acid and [^{3}H]arachidonic acid were made to have approx-

imately equal cpm in ^{14}C and ^{3}H. The concentration of arachidonic acid was adjusted with unlabelled arachidonic acid to be similar to that of the eicosapentaenoic acid. The measured amount of dual-labelled substrate added to each incubation provided 1.1×10^6 dpm [^{14}C]eicosapentaenoic acid and 1.4 or 1.7×10^6 dpm [^{3}H]arachidonic acid with the final concentration of each fatty acid averaging 2.4 μM.

Blood samples were drawn from the antecubital vein of human donors who had not taken anti-inflammatory drugs for the previous two weeks. Vacutainer collection tubes containing 1/10 volume ACD (citric acid, 0.8%; sodium citrate, 2.2%; dextrose, 2.45%; pH 4.5) as anticoagulant or blood collection bags containing 63 ml citrate phosphate dextrose adenine (2.0 g dextrose, 1.7 g sodium citrate, 206 mg citric acid, 140 mg monobasic sodium phosphate, 17 mg adenine) as anticoagulant were used. Platelets were isolated from the blood and washed by the method of McKean et al. (18). For the final suspension, platelets were suspended in a known volume of Tris/saline buffer (5 mM D-glucose, 0.15 M NaCl, 0.02 M Tris hydroxy methyl aminomethane, pH 7.4). Platelets were counted by phase contrast microscopy and the platelet concentration adjusted to $8\text{–}9 \times 10^5$ platelets/μl with the Tris/saline buffer. Three ml aliquots of platelet suspension were incubated at 37 C in a shaking water bath for 1 min. Immediately after 12 or 16 μl of dual-labelled substrate in ethanol was introduced, the platelet suspension was mixed gently on a vortex mixer and incubated for times from 0 to 5 min. On the basis of this time study, replicate incubations were subsequently carried out for 60 sec. Control samples consisted of platelet suspensions which were quenched with the lipid-extracting mixture just prior to the addition of the radioactive fatty acids. All reactions were stopped with chloroform/methanol/concentrated HCl (100:200:5, v/v/v), and the lipids were extracted by the modification of the Bligh and Dyer method (19) as described by Allen and Michell (20). After the chloroform phase was removed, the upper phase was re-extracted with chloroform and the two extracts combined. Control experiments with labelled arachidonic acid revealed that the average amount of radioactivity in PE in the acidified-Bligh and Dyer method was 10% lower compared to the standard method; this difference was found not to be statistically significant ($P = 0.11$).

In order to remove the unincorporated radiolabelled fatty acids, the phospholipids were separated from the neutral lipids by thin layer chromatography on activated silica gel H plates developed in heptane/isopropyl ether/acetic acid (60:40:3, v/v/v). The phospholipid band (origin) was scraped into tubes. After the addition of 2 ml water, the gel was eluted with 3.75 vols chloroform/methanol/concentrated HCl (100:200:5, v/v/v), and the acidified extraction method was followed as for platelets. Fifty percent of the extract, in the case of the time study, and 100%, in the case of the replicates, was then applied to activated silica gel H plates and developed in chloroform/methanol/acetic acid/water (50:30:0.5:3, v/v/v/v) for the separation of individual phospholipids. The lipid bands were detected under UV light after spraying with 2′,7′-dichlorofluorescein in methanol/water (50:50, v/v) and exposure to acetic acid vapor and then ammonia vapor. Lipid fractions were scraped into scintillation vials to which 1.5 ml water and then 13.5 ml Aquasol-2 (New England Nuclear Corp., Boston, Massachusetts) were added prior to counting using a Beckman LS7800 scintillation counter (Beckman, Irvine, California).

RESULTS

The extent of uptake of both [^{3}H]arachidonic acid and [^{14}C]eicosapentaenoic acid into total platelet phospholipid progressed steadily with increasing time, as shown in Figure 1. It is evi-

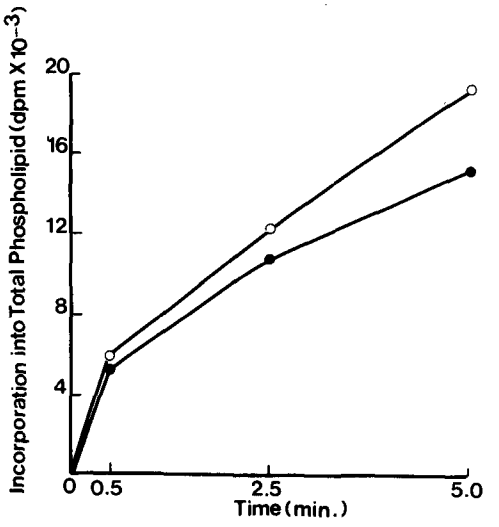

FIG. 1. The simultaneous incorporation of [^{3}H]-arachidonic acid and [^{14}C]eicosapentaenoic acid into total phospholipid (sum of PC, PS, PI, PE and sphingomyelin) of washed human platelets as a function of incubation time. Each incubation (3 ml) contained $8\text{–}9 \times 10^8$ platelets/ml and 1.7×10^6 dpm [^{3}H]arachidonic acid and 1.1×10^6 dpm [^{14}C]eicosapentaenoic acid at concentrations of 3.1 and 2.7 μM, respectively. O——O, [^{3}H]arachidonic acid; ●——●, [^{14}C]eicosapentaenoic acid.

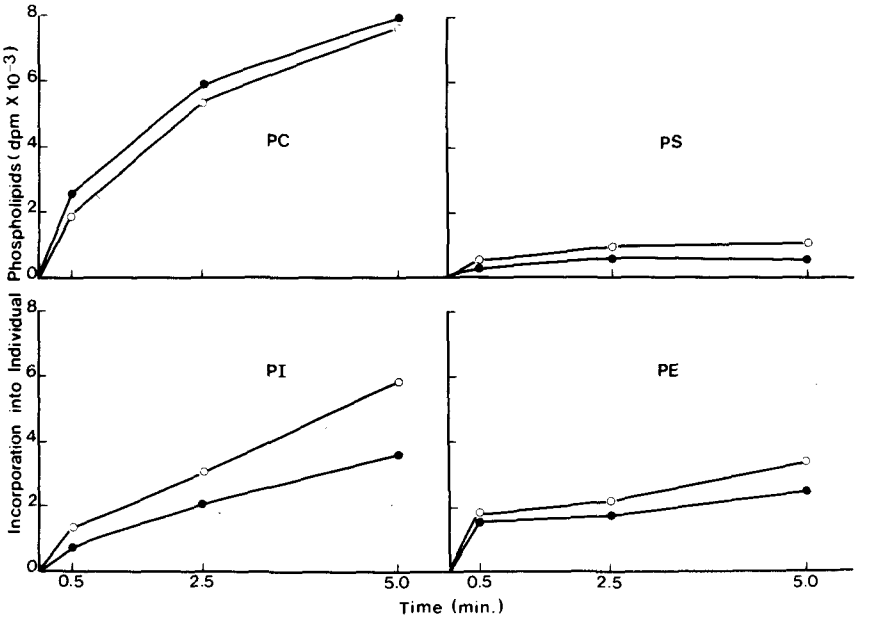

FIG. 2. The simultaneous incorporation of [^{3}H]arachidonic acid and [^{14}C]eicosapentaenoic acid into the individual phospholipids of washed human platelets as a function of incubation time. See legend to Fig. 1 for incubation conditions. Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine.

dent from the results shown in Figure 2 that there were differences in the incorporation of AA versus EPA among the individual phospholipids, and that these differences persisted with time up to 5 min. The entry of [^{14}C]EPA relative to [^{3}H]AA into PC appeared to be greater than that for PI.

A brief time of 60 sec was chosen for replicate incubations, over which period the incorporation phase was predominating. The percentage distribution of incorporated EPA among the individual platelet phospholipids was significantly different from that for AA (Table 1). The percentage of incorporated radiolabelled fatty acid into PC was greater for EPA than for AA ($p < 0.01$). In the case of PS and PI, the proportion of radiolabelled fatty acid was greater for AA than for EPA ($p < 0.01$). No difference in the proportion of radiolabelled fatty acid in PE was found when comparing AA and EPA.

When the [^{3}H]AA/[^{14}C]EPA dpm ratios were normalized to a substrate ratio of unity, examination of radioactivity appearing in the individual phospholipids indicated that individual phospholipids exhibit differences in the relative incorporation of AA versus EPA (Table 2). The [^{3}H]AA/[^{14}C]EPA ratio of newly incorporated fatty acid into PC and PE were significantly less ($p < 0.01$) than that for the substrate mixture, indicating a selectivity for EPA over AA.

TABLE 1

The Percentage Distributions of Incorporated [^{3}H]Arachidonic Acid and [^{14}C]Eicosapentaenoic Acid among Individual Platelet Phospholipids[a]

Phospholipid	[^{3}H]AA	[^{14}C]EPA	P value[b]
PC	43.3 ± 1.0	57.3 ± 1.0	$p < 0.01$
PS	8.8 ± 0.2	6.8 ± 0.1	$p < 0.01$
PI	32.6 ± 1.1	21.1 ± 0.9	$p < 0.01$
PE	15.2 ± 0.4	14.9 ± 0.4	n.s.

[a]The percentage distributions are given as mean values ±SE (n = 5). Abbreviations: AA, arachidonic acid; EPA, eicosapentaenoic acid; n.s., not significantly different from unity ($p > 0.05$); other abbreviations as given in legend for Fig. 2. Each incubation (3 ml) contained 8-9 × 10^8 platelets/ml and 1.4 × 10^6 dpm [^{3}H]arachidonic acid and 1.1 × 10^6 dpm [^{14}C]eicosapentaenoic acid at concentrations of 2.0 and 1.9 μM, respectively.

[b]The percentage distributions for [^{14}C]EPA were tested for significant differences relative to those for [^{3}H]AA by the Student's t test (21).

In contrast, the corresponding ratio in PI was significantly greater ($p < 0.01$) than unity. No significant difference in the case of PS was observed in the incorporation of AA versus EPA. In the case of total phospholipid, relatively more EPA than AA was incorporated, as found for PC and PE.

TABLE 2

The Relative Incorporation of [^{3}H]Arachidonic Acid versus [^{14}C]Eicosapentaenoic Acid into Human Platelet Phospholipids[a]

Phospholipid	Ratio [^{3}H]AA/[^{14}C]EPA	P value[b]
PC	0.55 ± 0.22	$p < 0.01$
PS	0.93 ± 0.04	n.s.
PI	1.12 ± 0.04	$p < 0.01$
PE	0.74 ± 0.03	$p < 0.01$
Total PL	0.71 ± 0.03	$p < 0.01$

[a]The ratios are given as mean values ±SE (n = 5) and calculated relative to the isotopic ratio in the corresponding substrate mixture (arbitrarily assigned a value of 1.00). See legend to Table 1 for incubation conditions. Abbreviations PL, phospholipid; other abbreviations as given in legend to Table 1.

[b]The ratios in the phospholipids relative to the substrate (1.00) were tested for significant differences by the Student's t test.

DISCUSSION

The present results indicate that, when mixtures of [^{3}H]AA and [^{14}C]EPA are presented to intact human platelets, more radioactive EPA is incorporated into PC than other platelet phospholipids (Fig. 2, Table 1); this result is in agreement with the mass distribution of EPA among the platelet phospholipids of human subjects following the consumption of fish oil (8). However, in the case of radioactive AA incorporation, a predominant incorporation into PC, as observed herein, is not in agreement with mass data (8) which indicate that PE contains more AA (in $\mu g/10^9$ platelets) than other individual phospholipids. Although not studied directly herein, Rittenhouse-Simmons et al. (22) have reported that labelled AA is preferentially incorporated into diacyl PE and not into plasmalogenic PE. In contrast to previous work where the incorporation of [^{14}C]AA was studied alone and provided a three-fold greater incorporation into PC as compared to PI (23–25), the present work with mixtures of [^{3}H]AA and [^{14}C]EPA indicated that the incorporation into PC was only moderately greater than that into PI (Table 1). The much greater incorporation of [^{14}C]EPA into PC as compared to PI, as observed herein (Table 1), has been reported by other investigators using platelet rich plasma (25,26) or washed platelet suspensions (24). Competition between the two polyunsaturated fatty acids, when presented as a mixture as employed in our investigations and the use of short time periods (1 min), may contribute to the difference in distributions observed herein as compared to that reported by other investigators studying fatty acids singly. In this regard, it is noteworthy that the fatty acid selectivities of many lipid-synthesizing enzyme systems are much more apparent when mixtures of the appropriate precursors are employed compared to when such precursors are studied singly (27). Furthermore, the use of mixtures in such experiments might better reflect physiological conditions.

The ratios of [^{3}H]AA/[^{14}C]EPA found in individual human platelet phospholipids (Table 2) were lowest for PC and highest for PI with the order being PC<PE<PS<PI. Interestingly, the mass ratios of AA/EPA found in human platelet phospholipids resulting from the consumption of fish oil followed the same trend (8,11). These results suggest that the relative incorporation of these exogenous polyunsaturated fatty acids may be partly responsible for controlling the relative amounts of AA and EPA in the individual platelet phospholipids resulting from the consumption of fish and/or fish oils.

The biochemical mechanisms accounting for the preferential incorporation of EPA (over AA) into PC and PE, and the preferential entry of exogenous AA (over EPA) into PI are of interest (Table 2). The polyunsaturated fatty acids generally are considered to enter mammalian phospholipids mainly via acylation of endogenous monoacyl phospholipids rather than via de novo synthesis (28). In this regard, our results generally are consistent with those of Inoue et al. (29), who observed that EPA-CoA was a better substrate than AA-CoA for the pig platelet microsomal acyl-CoA:1-acyl-*sn*-glycero-3-phosphocholine acyltransferase forming PC while, in contrast, AA-CoA was a better substrate than EPA-CoA for the acyl-CoA:1-acyl-*sn*-glycero-3-phosphoinositol acyltransferase. However, Iritani and colleagues (30), using rat platelets, reported moderately higher acyltransferase rates in PC synthesis when AA-CoA rather than EPA-CoA was used as a substrate. The relative utilization of mixtures of AA-CoA and EPA-CoA for the phospholipid acyltransferases in human platelet preparations remains to be investigated. Both EPA and AA have been found to be excellent substrates for an acyl-CoA synthetase in human platelets which readily utilizes eicosanoid precursors (31–33).

In conclusion, these experiments indicate that human platelets exhibit significant selectivity in the relative incorporation of AA versus EPA into individual phospholipids when polyunsaturated fatty acids are presented as

mixtures. These selectivities may account in part for the differing AA/EPA mass ratios that have been observed among the individual phospholipids of human subjects consuming fish oils.

ACKNOWLEDGMENT

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METHODS

Analysis of Positional Distribution of Fatty Acids in Palm Oil by ^{13}C NMR Spectroscopy

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ABSTRACT

The ^{13}C NMR spectrum of the carbonyl carbons of the acyl groups of triacylglycerols of palm oil has been shown to give the composition of saturated, oleic and linoleic acyl groups at the 1,3-positions and at the 2-position of the glycerol moiety. Except for the lack of differentiation of the saturated fatty acids, the ^{13}C NMR technique provides the same information as the tedious enzymatic hydrolysis cum fatty acid analysis. The carbonyl carbon of the linolenic acyl group (18:3,[*cis,cis,cis*]-9,12,15) has a chemical shift which is only 0.005 ppm to low frequency of that of the linoleic acyl group (18:2,[*cis,cis*]-9,12), so that the two resonances may not be distinguishable (or resolved) even at a high magnetic field.
Lipids 20:778–782, 1985.

INTRODUCTION

Recently we have shown that in the ^{13}C NMR spectrum of the carbonyl carbons of the triacylglycerols of palm oil, the carbonyl carbons of the saturated, oleic and linoleic acyl groups at the 1,3-glycerol positions have slightly different chemical shifts, and the same is true for the acyl groups at the 2-glycerol position (1). At low magnetic field ($\leq$2.3T), the resonance peaks are not well resolved because of overlap; only qualitative information about the composition of the fatty acids at the two glycerol positions could be obtained. However, at higher magnetic fields, the peaks can be resolved and quantitative information can be obtained from the integrals of the peaks.

The conventional method of obtaining the composition of fatty acids at the two glycerol positions of triacylglycerols involves hydrolysis by pancreatic lipase which preferentially removes the acyl groups attached to the 1,3-glycerol positions, leaving a 2-monoacylglycerol (2). In this study, we show that essentially the same information can be obtained from the ^{13}C NMR spectrum of the carbonyl carbons of the acyl groups of the triacylglycerols of palm oil (or any vegetable oil or fat).

Previous studies of ^{13}C NMR as a quantitative analytical method for determining the composition of fats and oils have focused on the aliphatic and olefinic carbons of the acyl chains (3–7). The method also has been applied to the analysis of oil composition in intact oilseeds (8,9). Except for determining the primary isomeric positioning of the butyryl group in butter oil (5), the resonances of the aliphatic carbons are not amenable to elucidation of the positional distribution of fatty acids in triacylglycerols. This is because the aliphatic carbons either have identical (i.e. not distinguishable) chemical shifts for a specific acyl chain at the two glycerol positions, or, when the chemical shifts are different for the two glycerol positions, have identical chemical shifts for all the fatty acids at a given glycerol position. However, it has been shown that the high field spectrum of the olefinic carbons can be used to determine the positional distribution of the oleic and linoleic acyl chains (10).

EXPERIMENTAL

The sample of natural palm oil used in this work was obtained from a local palm oil mill. The synthetic model triacylglycerols: tripalmitin (16:0), triolein (18:1,[*cis*]-9), trilinolein (18:2, [*cis,cis*]-9,12) and trilinolenin (18:3,[*cis,cis,cis*]-9,12,15) were obtained from Sigma Chemical Co., St. Louis, Missouri, and were used without further purification.

The ^{13}C NMR spectrum in Figure 2 was recorded on a JEOL GX270 FT-NMR spectrometer operating at 67.70 MHz. The 25.05 MHz ^{13}C NMR spectra were recorded on a JEOL FX100 FT-NMR spectrometer. The spin-lattice relaxation times were measured with the latter instrument, using the inversion-recovery method. The samples in $CDCl_3$ solution were degassed through five freeze-pump-thaw cycles.

Some experimental conditions found necessary for achieving good resolution in the spectrum of the carbonyl carbons of palm oil are: (i) fairly dilute solution in $CDCl_3$ (concentration 1:4, v/v or 2 mol %) and preferably

filtered, and (ii) higher sample temperature at high magnetic field than at low field, e.g. 50 C at 67.70 MHz versus 28 C at 25.05 MHz. In a superconducting magnet it is easier to attain good spectral resolution with a long sample (~5 cm).

Where integrals of the resonance lines are needed, caution should be used when resolution enhancement is applied in processing the accumulated digital data as it can distort areas if the lines do not have the same width. The reliability of quantitating the carbonyl carbon resonances depends on the carbons having identical NOE and similar T_1 values. Provided that the T_1 values do not differ significantly, the error that may arise in the quantitation can be minimized by using a small pulse angle, such as 30° or less, and a pulse repetition time which exceeds the longest T_1 value.

RESULTS AND DISCUSSION

The carbonyl carbons of the saturated, oleic and linoleic acyl chains of the triacylglycerols of palm oil have different chemical shifts but identical nuclear Overhauser enhancement (NOE) (1). The assignment of these chemical shifts is confirmed by the ^{13}C NMR spectrum of the carbonyl carbons of synthetic model triacylglycerols shown in Figure 1. The carbonyl carbons of copolyesters of terephthalate and diols are also found to have identical NOE (11). This result provides confirmation for the observation in palm oil. Therefore, the proton noise decoupled ^{13}C NMR spectrum of the carbonyl carbons in palm oil (or any vegetable oil or fat) can be used for quantitative analysis of the fatty acid composition at the glycerol positions if the resonance peaks are sufficiently well resolved.

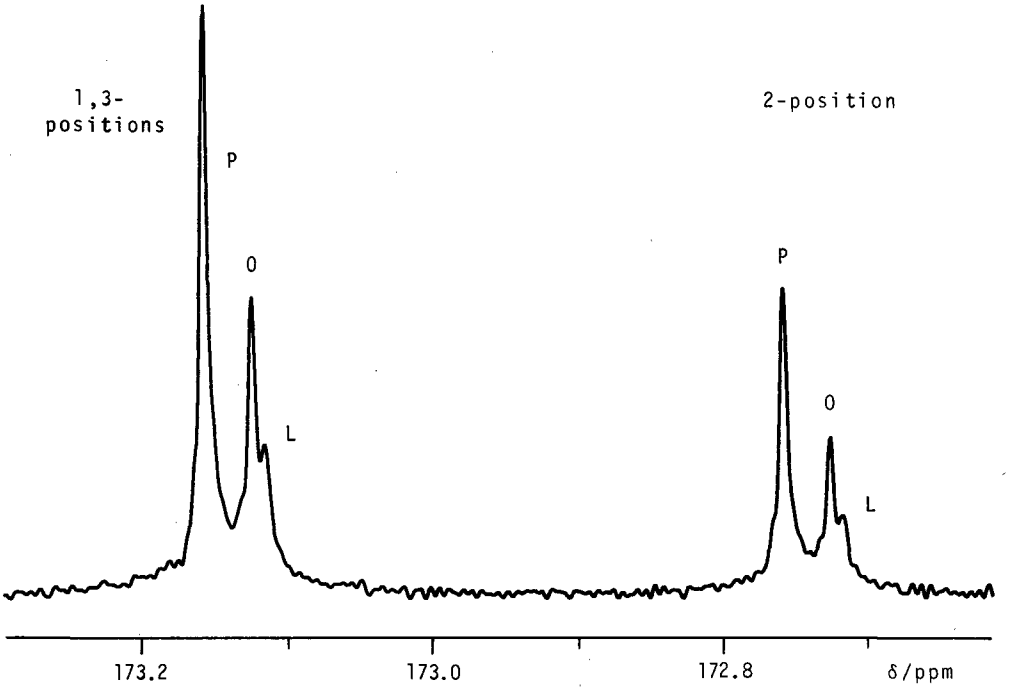

FIG. 1. Low field (25.05 MHz) proton noise decoupled ^{13}C NMR spectrum of the carbonyl carbons of a mixture of 3 synthetic model triacylglycerols in chloroform-*d* solution at 28 C with the following concentrations: tripalmitin, 1.5 mol %; triolein, 1.0 mol %, and trilinolein, 0.5 mol %. The peaks are assigned to the acyl groups at the two glycerol positions as follows: P (palmitic); O (oleic), and L (linoleic). The integrals of the peaks at the 1,3-positions are 2.0 times those at the 2-position. The chemical shifts of the peaks for the 1,3-positions and 2-position are: palmitic, δ 173.160 and 172.759; oleic, δ 173.127 and 172.721, and linoleic, δ 173.118 and 172.712, respectively. The spectrum was obtained with the following parameters: data points 8 K (zero-filled to 16 K), spectral width 200 Hz, pulse angle 30°, acquisition time 7.68 s, and pulse delay 0.5 s. With sample tube size 10 mm OD, this spectrum is the result of 1510 scans, requiring a total accumulation time of 206 min. The spectrum was resolution enhanced with an exponential factor of −8 (or line narrowing 0.0310 Hz).

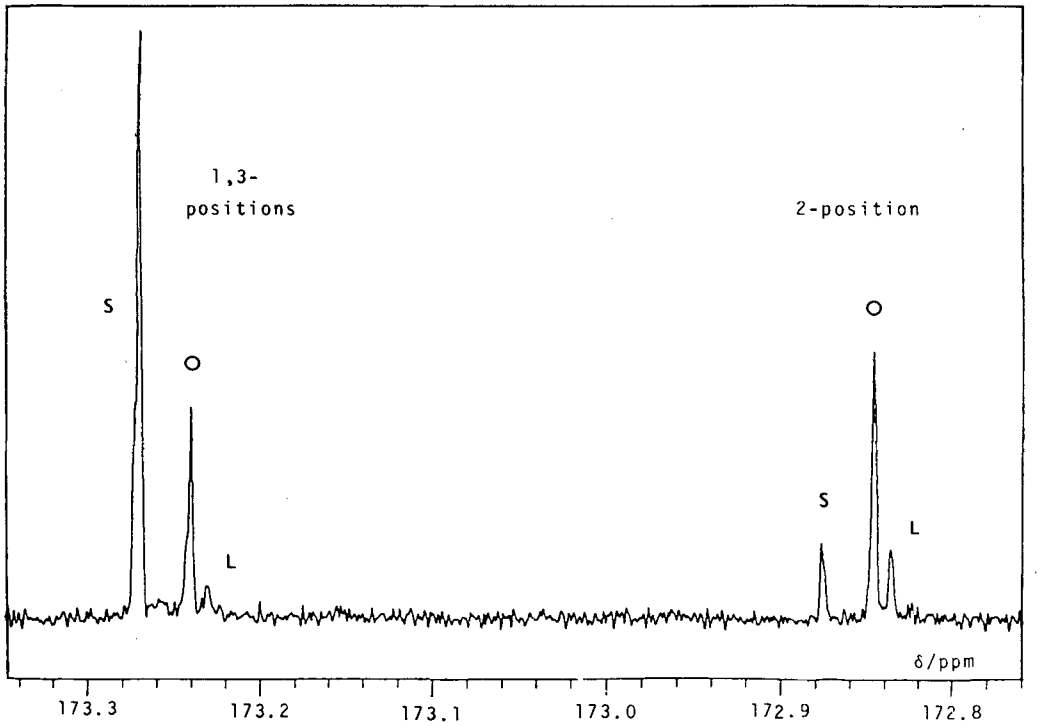

FIG. 2. Medium field (67.70 MHz) proton noise decoupled ^{13}C NMR spectrum of the carbonyl carbons of the triacylglycerols of palm oil in chloroform-*d* solution (concentration 1:4, v/v) at 50 C. The peaks are assigned to the acyl groups at the two glycerol positions as follows: S (saturated), O (oleic), and L (linoleic). The integrals of the peaks at the 1,3-positions are 2.0 times those at the 2-position. The spectrum was obtained with the following parameters: data points 32 K, spectral width 800 Hz, pulse angle 45°, acquisition time 10.2 s, and pulse delay 0.2 s. With sample tube size 10 mm OD, this spectrum is the result of 256 scans, requiring a total accumulation time of 45 min. The spectrum was resolution enhanced by a Gaussian transformation.

The medium magnetic field (67.70 MHz) ^{13}C NMR spectrum of the carbonyl carbons of a sample of palm oil is shown in Figure 2, in which the resonance peaks are sufficiently well resolved. The integrals of the peaks for the 1,3-glycerol positions are exactly twice those for the 2-glycerol position, indicating that the resonances have identical NOE, as in the case of low magnetic field. The NOE is not expected to vary much at higher magnetic fields because it arises largely from the magnetic dipole-dipole relaxation mechanism, which is not dependent on the magnetic field (12). The spin-lattice relaxation times of the carbonyl carbons, however, are expected to vary (decrease) at higher magnetic fields because of contributions from the chemical shift anisotropy (CSA) relaxation mechanism, which is highly field-dependent (12). Therefore, the integrals of the peaks in the spectrum in Figure 2 give the composition of fatty acids in the sample of palm oil (Table 1). Table 2 shows an example of fatty acid composition of palm oil obtained by enzymatic hydrolysis cum fatty acid analysis (13). The data in Table 1 for one sample of palm oil are within the range of variation of fatty acid composition of natural palm oil and are comparable with the data in Table 2.

TABLE 1

Fatty Acid Composition of Palm Oil Determined by ^{13}C NMR of the Carbonyl Carbons[a,b]

Acyl group	1,3-Positions	2-Position	Total
Saturated	73.4	18.8	55.1
Oleic	23.0	64.6	36.9
Linoleic	3.6	16.6	8.0

[a]In mole %.
[b]Uncertainty: ±0.5%.

With the data in Table 1, the 1,3-random–2-random distribution theory, which was sug-

gested independently by Vander Wal, Coleman and Fulton, and Gunstone (14-16), may be used to calculate the composition of the various species of triacylglycerols that differ in the positional distribution of fatty acids on the glycerol moiety (17). However, the validity of this random distribution theory for palm oil has not been proven.

A shortcoming of this ^{13}C NMR technique is that at present there is no differentiation of the various saturated fatty acids of palm oil, which are palmitic (~44%), stearic (~4.5%) and myristic (~1%). In addition, as Figures 1 and 3 show, it may not be possible to distinguish between linoleic and linolenic acyl groups from the spectrum of the carbonyl carbons. The linolenic carbonyl carbon has a chemical shift which is only 0.005 ppm to low frequency of that of the linoleic carbonyl carbon. If appreciable quantities of both these acyl groups are present, the two resonances may not be resolved, even at high magnetic field; a slightly broadened peak or one with a slight shoulder would be observed. Because of the low sensitivity of the carbonyl carbon, the presence of a trace amount of an acyl group may not be detectable in the NMR spectrum.

If and when the resonance peaks for the oleic and linoleic carbonyl carbons cannot be well enough resolved for a reliable integral of each peak to be obtained, the total integral for both peaks should be obtained. Then a high resolution spectrum at high magnetic field of the olefinic carbons should be acquired under conditions appropriate for quantitative analysis. This spectrum gives the distribution of oleic and linoleic acyl groups at the two glycerol positions (10), and their relative proportions can be obtained therefrom. The composition of the two unsaturated acyl groups can then be calculated. The information from the spectrum of the olefinic carbons may be used to check the result derived from the spectrum of the carbonyl carbons even in cases when good resolution in the latter is achieved. Should linolenic acyl group be present, its proportion relative to those of linoleic and oleic can be determined from the spectrum of the olefinic carbons, and the procedure described above can be extended to obtain its composition.

TABLE 2

An Example of Fatty Acid Composition of Palm Oil Obtained by Enzymatic Hydrolysis cum Fatty Acid Analysis[a,b]

Acyl group	2-Position	Total
Saturated	19.1	49.6
Oleic	60.5	39.2
Linoleic	19.7	10.1
Others	0.7	1.1

[a]In mole %.

[b]Source of data: Reference 13.

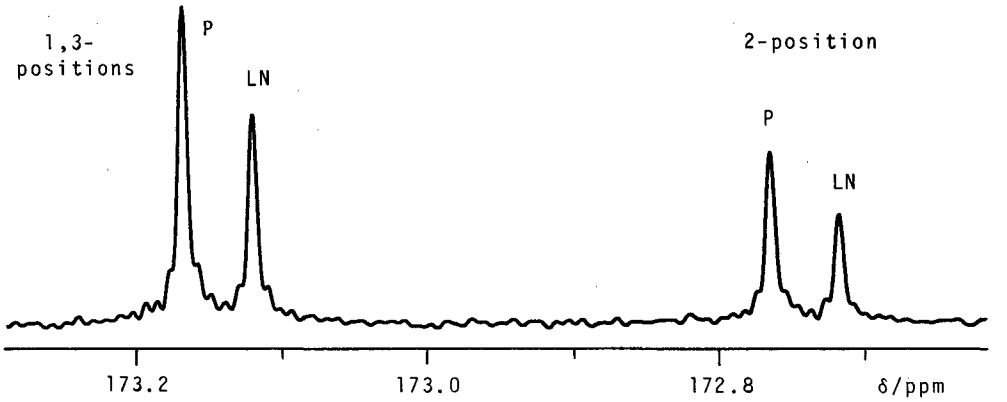

FIG. 3. 25.05 MHz proton noise decoupled ^{13}C NMR spectrum of the carbonyl carbons of a mixture of synthetic tripalmitin and trilinolenin in chloroform-*d* solution at 28 C with the following concentrations: tripalmitin 1.0 mol % and trilinolenin 0.7 mol %. The peaks are assigned to the acyl groups at the two glycerol positions as follows: P (palmitic) and LN (linolenic). The chemical shifts of the peaks for the 1,3-positions and 2-position are: palmitic, δ 173.170 and 172.764; and linolenic, δ 173.123 and 172.718. Thus, the carbonyl carbons of linoleic (18:2) and linolenic (18:3) acyl groups have practically identical chemical shifts (cf. Fig. 1). The spectrum was obtained with the following parameters: data points 8 K (zero-filled to 16 K), spectral width 200 Hz, pulse angle 30°, acquisition time 5.12 s, and pulse delay 1.0 s. With sample tube size 10 mm OD, this spectrum is the result of 1072 scans, requiring a total accumulation time of 110 min. No resolution enhancement was applied. The ratio of the integrals of the peaks for the two glycerol positions is 2.0.

TABLE 3

Spin-Lattice Relaxation Times (T_1/s) of Selected Carbons of Model Triacylglycerols (3.0 Mol %) in $CDCl_3$ Solution at 28 C and 25.05 MHz[a,b]

Triacylglycerol	C-α	C-β	C-1 (α)	C-1 (β)	C-9[c]	C-10	C-12	C-13	C-15	C-16	C-17	C-18
Tripalmitin	0.20	0.33	7.6	6.9				2.0	3.7	4.6		
Triolein	0.20	0.31	8.8	7.6	1.5	1.6			2.1	3.1	4.0	4.7
Trilinolein	0.21	0.34	9.8	8.9	2.0	2.0	3.2	3.2	3.1	4.1	4.8	5.4

[a]α and β refer to the 1.3-positions and 2-position, respectively; C-α and C-β are the glycerol carbons, and C-1 is the carbonyl carbon.

[b]Uncertainties: for C-α and C-β, ±0.02 s; C-1, ±0.5 s; other carbons, ±0.2 s or less.

[c]The values shown for C-9 pertain to the 1,3-acyl chains; the value for C-9 of the 2-acyl chain is 0.1–0.2 s less. For the other olefinic carbons only one value is obtained as the peaks for the two glycerol positions were not resolved.

The spin-lattice relaxation times T_1 of the glycerol and selected carbons in the acyl chains of model triacylglycerols are given in Table 3. These data may be useful in selecting parameters for the acquisition of ^{13}C NMR spectra of oils and fats. At high magnetic field the T_1 values of the carbonyl carbons are expected to be reduced substantially through the CSA relaxation mechanism and become more similar in magnitude. The T_1 values of the carbonyl carbons, even at low field, can be considered low relative to those of the protonated carbons at the free end of the acyl chain. The extremely low T_1 values of the glycerol carbons, together with those of the carbonyl carbons, indicate that the central portion of the triacylglycerol molecule is relatively immobile. The long correlation time, τ_c, associated with this slow motion implies that the "extreme narrowing condition," $\omega_o\tau_c \ll 1$, where ω_o is the Larmor frequency (12), may not hold at high magnetic field. Consequently, to obtain narrow lines in the spectrum of the carbonyl carbons at high field, a higher sample temperature is required than at low field (see Experimental section).

The addition of a relaxation reagent such as $Cr(acac)_3$ to solutions of oils in $CDCl_3$ shortens the T_1 values of the aliphatic carbons of the acyl chain (3). However, it is found that the presence of this relaxation reagent in $CDCl_3$ solutions of palm oil broadens the resonance lines of the carbonyl carbons and thereby ruins the resolution.

ACKNOWLEDGMENTS

The Application Division of JEOL Ltd., Tokyo, provided the spectrum in Figure 2. B. K. Tan of the Palm Oil Research Institute of Malaysia furnished the data in Table 2; Richard B.H. Quah gave technical assistance, and the University of Malaya provided financial support.

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HPLC of Triglycerides with Gradient Elution and Mass Detection

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ABSTRACT

A gradient elution reversed phase HPLC system with a mass detector was investigated for the separation of triglycerides. A gradient based on a ternary eluent mixture is suggested. The detector was investigated in terms of linearity, reproducibility and relative response. The separation of soybean oil and some other natural triglyceride mixtures is demonstrated.
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INTRODUCTION

The separation of triglycerides has undergone a dramatic change during the last decade. The utilization of highly efficient chromatographic columns has made it possible to separate triglyceride mixtures into well-defined species or groups of species. Both gas and liquid chromatography have benefited from the development of such columns.

High performance liquid chromatography (HPLC) has been used for triglyceride separations almost exclusively in the reversed phase mode, i.e. in connection with bonded phase C18 packing material (1-4). Generally, isocratic conditions in combination with RI and UV detectors were reported (5). The complexity of natural triglyceride mixtures would, however, require gradient elution to fully utilize the resolution power of the liquid-liquid chromatographic systems. This is not possible to achieve in combination with RI detectors and difficult with UV detectors in this case due to the short wavelengths that have to be used. The reported uses of gradient elutions were connected with halogenated triglycerides or special, home-constructed detectors (6-8).

The introduction of the commercially available mass detector meant an expansion of the possible conditions for triglyceride separations, including that of gradient elution. The basic principle of this detector, i.e. the light scattering, originally was presented by Charlesworth (9). The commercial version has been available for some years now and is used by several groups.

This work was carried out to investigate the possibilities of using a gradient system for triglyceride separation. Preliminary tests showed that the mass detector gave very narrow and symmetrical peaks which indicated its usefulness for this application. The goal was to find a system that could be used to separate any natural triglyceride mixture regardless of its carbon number distribution. The present study represents our first general results concerning the use of the mass detector in this sense.

During the course of this work, two reports from the same research group regarding the use of mass detection of triglycerides appeared in the literature. Tsimidou and Macrae (10) reported on the influence of the injection solvent, and Robinson and Macrae (11) compared different detection systems, both works applied on reversed phase separation of triglycerides.

MATERIALS AND METHODS

The liquid chromatographic system consisted of a Shimadzu LC 4A ternary gradient flow system equipped with SIL-2AS autosampler (Kyoto, Japan). The column (250 × 4.6 mm) was packed with Lichrospher RP 100 (Merck AG, Darmstadt, West Germany) and thermostatted at 22 C. The mass detector (Applied Chromatography Systems Ltd., Luton, Bed., U.K.) oven temperature was 40 C and the inlet gas pressure (from an air compressor) 15 psi.

The mobile phase consisted of mixtures of acetonitrile (Rathburn, Walkerburn, U.K., Grade S), ethanol (spectroscopic grade) and hexane (Merck AG, spectrographic grade). Gradients from 0 to 100% of ternary premixtures of the three solvents were used at a flowrate of 1 ml/min.

The triglyceride mixtures used in this work were common, refined oils and a commercial standard (HPLC G-1, Nu-Chek Prep, Inc., Elysian, Minnesota). The sample was dissolved in hexane:isopropanol (1:1, w/w) and 12 μl were injected (approx. 150 μg total sample).

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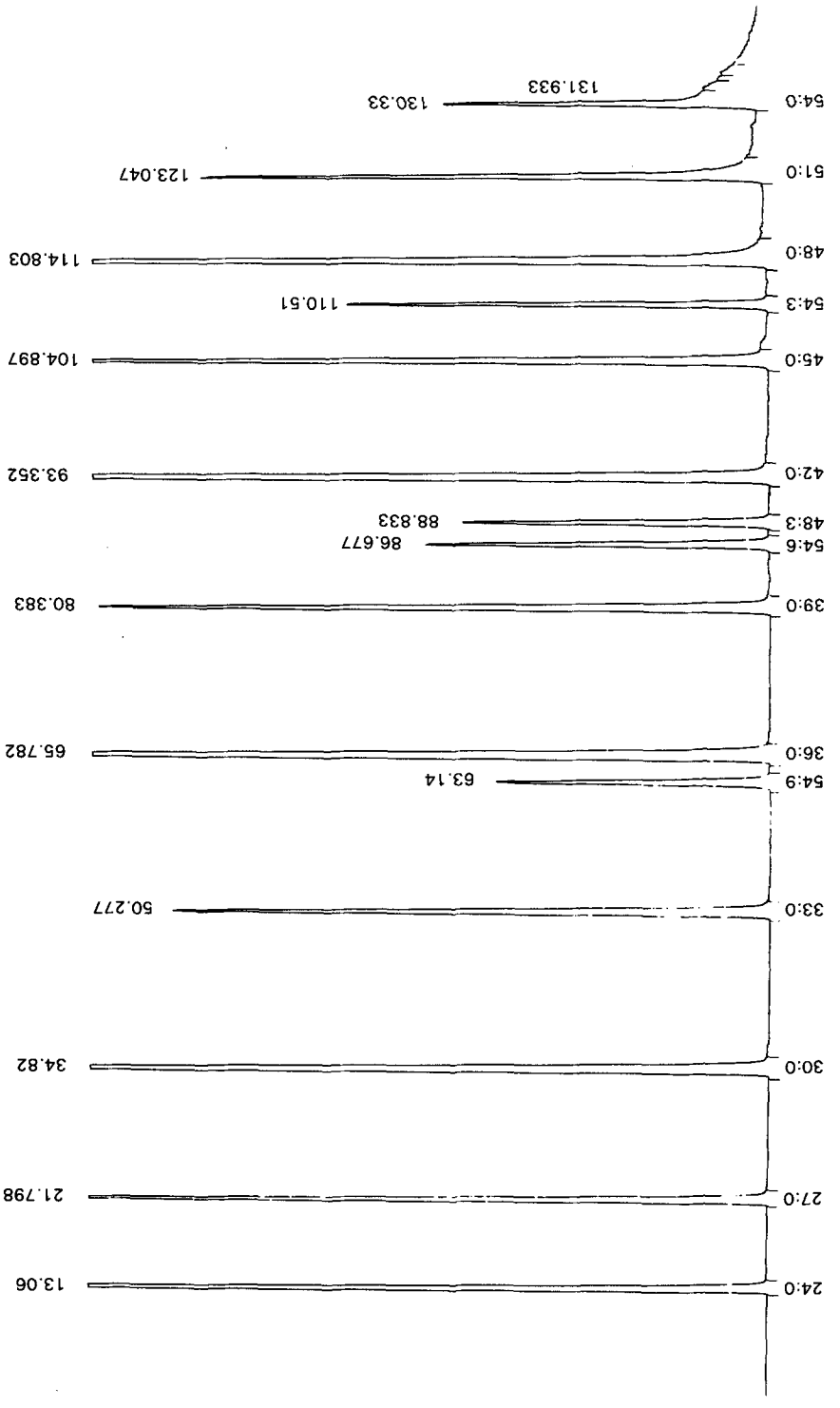

FIG. 1. Separation of a standard triglyceride mixture (G No. 1, Nu-Chek Prep). Linear gradient from 100% A to 100% B in 120 min. Column, 250 × 46 mm LiChrospher 100 RP 18; Solvent A, acetonitrile; Solvent B, acetonitrile-ethanol-hexane (40:40:20, w/w/w); flowrate, 1 ml/min.

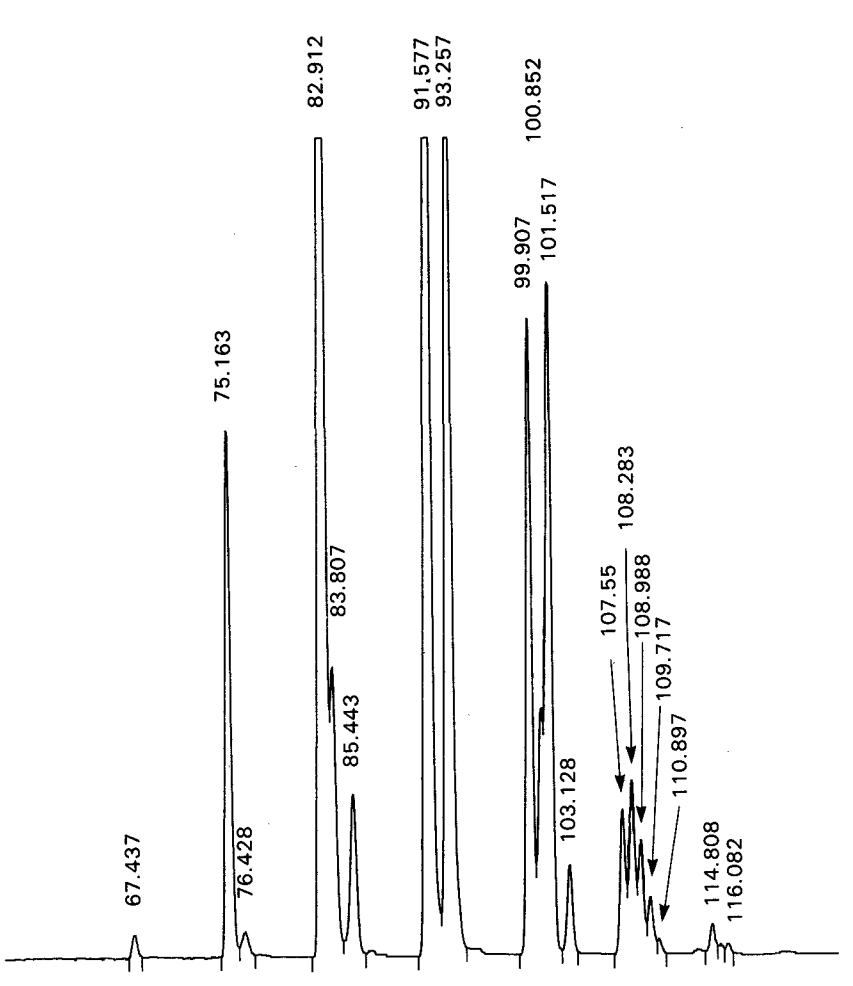

FIG. 2. Separation of soybean oil triglycerides. Chromatographic conditions as in Fig. 1.

RESULTS

Solvent Program

By initially investigating different gradient profiles, we concluded that it was most practical to use a linear gradient, also taking into account the possibility of calculating the equivalent carbon numbers (ECN-values) of the obtained peaks.

Solvent A (100% acetonitrile) was then replaced gradually by the ternary mixture B (acetonitrile + ethanol + hexane). The composition of B determines the selectivity and elution power. By a proper selection of proportions, most natural triglyceride mixtures can be resolved into peaks that represent species with at least 0.1 units difference expressed as ECN-values (cf. Table 2 and Fig. 1 regarding the separation of 54:6 and 48:3 in comparison to the natural mixtures).

Triglyceride Mixtures

The chromatogram of the standard mixture of triglycerides is shown in Figure 1. The separations of soybean oil and a mixture of soybean and coconut oils illustrate the resolution of vegetable oils into triglyceride species (Figs. 2 and 3).

A sample of extracted fat from Swedish butter (Fig. 4) demonstrates the complexity of animal fats.

Reproducibility

The reproducibility of the triglyceride representation of soybean oil was investigated

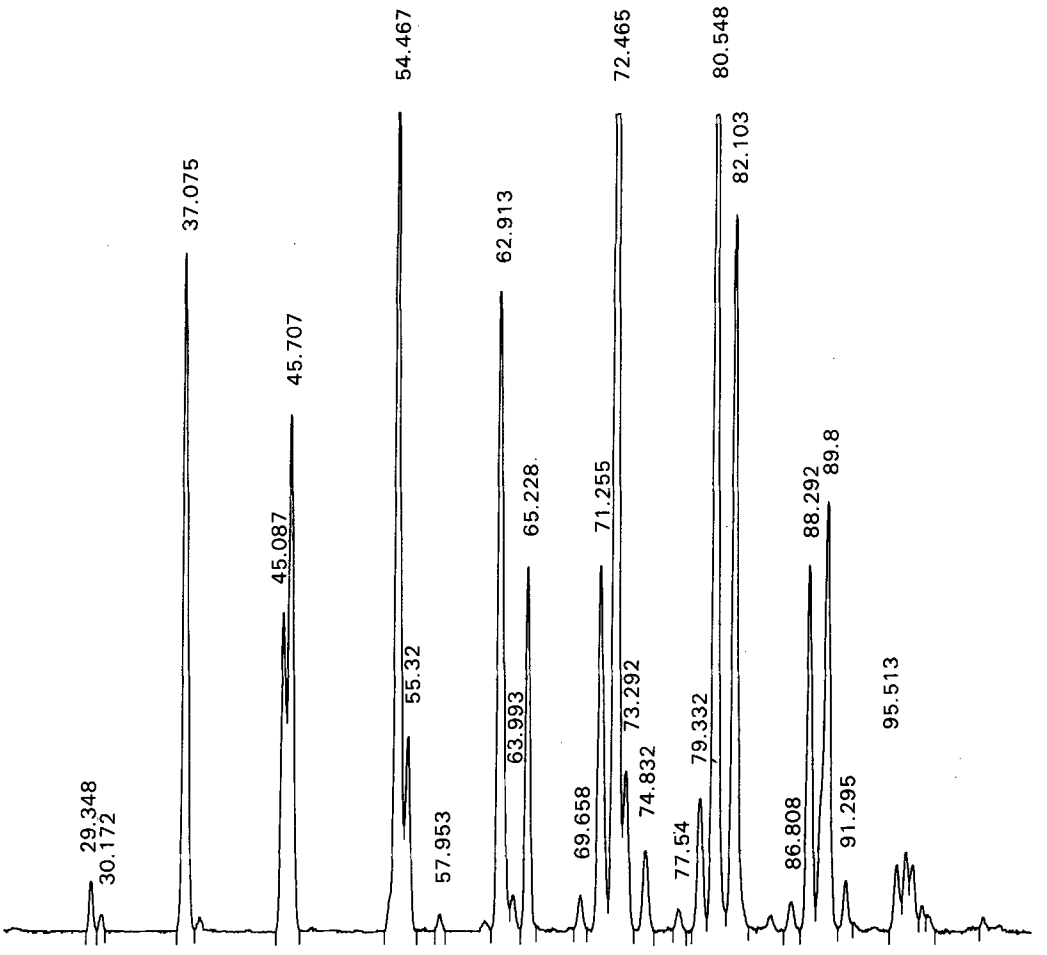

FIG. 3. Separation of a mixture of equal amounts of soybean oil and coconut oil. Chromatographic conditions as in Fig. 1.

by repeated injections (Table 1). The obtained relative standard deviations (RSD-values) are as reproducible as the values from other, more commonly used HPLC detectors. The largest peaks in soybean oil (>4 area%) had RSD-values of less than 2%, which is satisfactory for most applications.

Quantitation

The area representation of the standard mixture (Fig. 1) is shown in Table 2. The slight over-representation of the larger peaks compared to the slightly under-represented smaller peaks indicates the non-linearity of the detector. However, the representation is much closer to the nominal values than the one obtained from the UV detector, which suffers from a strong over-representation of the unsaturated triglycerides.

The non-linear behavior of the mass detector was more apparent when amount/response studies were carried out. One example is shown in Figure 5, in which the response of different amounts of trioctanoin is plotted. Linear regression could be used as a fairly good approximation, but the exponential curve obtained by the computer (HP 85 Power Curve program) reflects much better the quantitative behavior of the detector. Results from similar plots of other triglycerides are presented in Table 3.

Detection Limit

The detection limit was estimated in combination with the quantitative studies. Figure 6 shows the chromatographic representation of

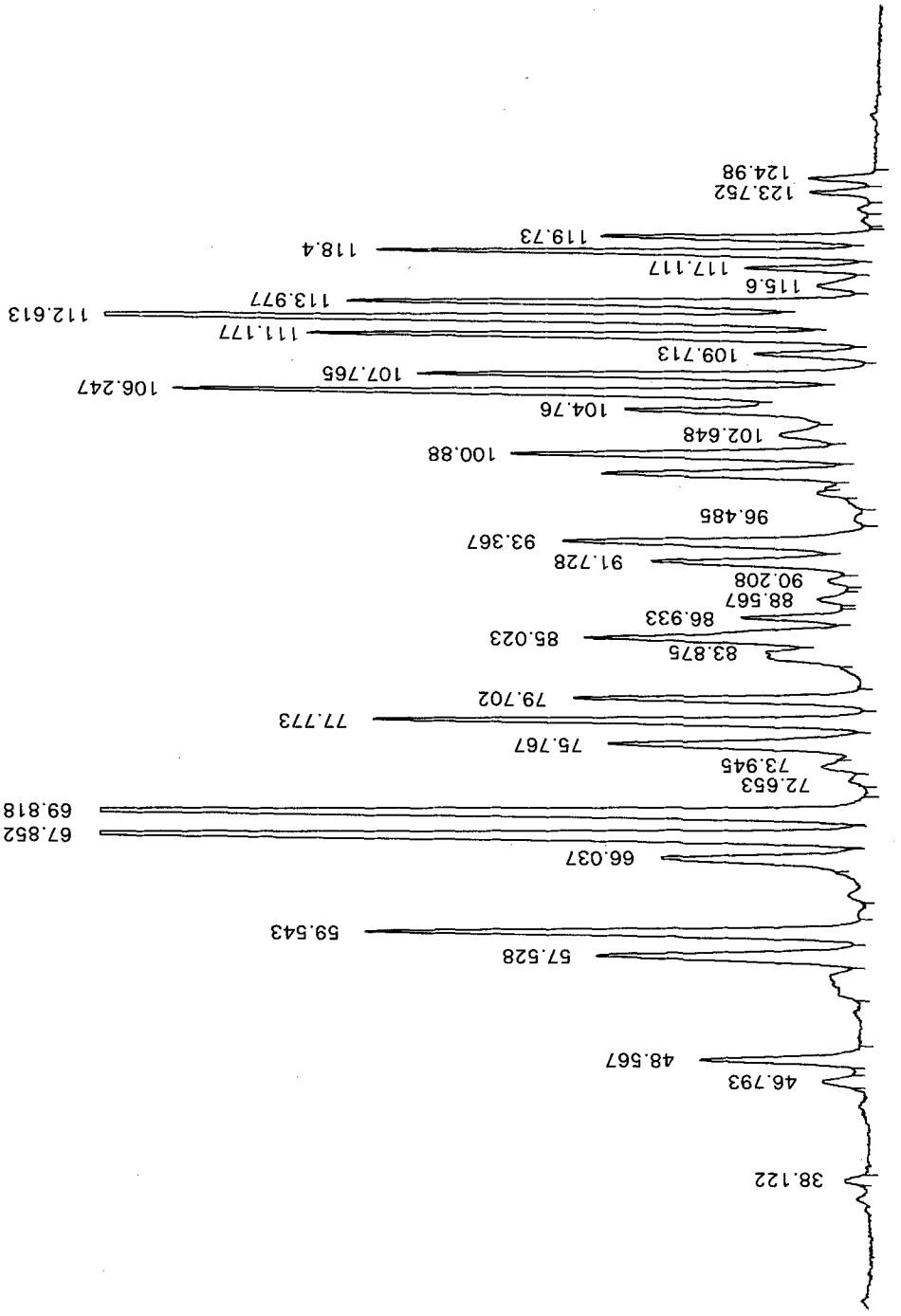

FIG. 4. Separation of fat extracted from butter. Chromatographic conditions as in Fig. 1.

the smallest injected amount of trioctanoin. From this the detection limit could be approximated to 1 μg.

DISCUSSION

The ternary mixture used (i.e. acetonitrile, ethanol and hexane) represents one of many possibilities to obtain good resolutions of the triglyceride mixtures. However, by changing the ratio of ethanol to hexane (in the pre-mixed B solvent) a wide range of mobile phase selectivity could be obtained. This will affect mainly the unsaturated triglycerides, thus resulting in different retention behavior of the so-called critical pairs. The peak capacity of the utilized gradient system would allow 50–100 triglyceride species to be separated; by modifying the mobile phase and the gradient slope, separation of specific oils and fats could be optimized. We feel that this enables us to identify the triglyceride species by retention data only, i.e. critical pairs in the traditional meaning are no longer "critical." In our opinion the time has come to identify new "critical" species and to work with ECN-values and solvent selectivity in the same way as fatty acid methyl esters are separated by different columns in gas chromatography.

The advent of the mass detector has introduced the possibility of the gradient elution for triglyceride separations by means of a commercially available detector. The separation of soybean oil triglycerides (Fig. 2) and the separation of the commercial mixture (Fig. 1) are practically identical to the corresponding chromatograms obtained by Privett et al. (12,6), who utilized their home-constructed FID-detector.

Our preliminary validation results indicate the usefulness of the mass detector in the

TABLE 1

Reproducibility Studies of the Relative Distribution of Soybean Oil Triglycerides[a]

Relative distribution (area%)	Reproducibility (n = 6) (RSD%) System[b]	Method[c]	Equivalent carbon number
25.2	1.1	1.1	41.0
24.9	1.2	0.6	42.6
14.6	1.7	1.6	42.9
11.9	1.8	1.4	44.9
8.5	1.9	1.2	44.5
4.7	1.6	1.8	39.5
2.4	3.4	5.2	41.2
2.0	6.9	8.3	47.0
1.8	9.3	4.9	46.6
1.3	6.4	6.6	41.4
1.2	8.3	11.5	47.2

[a]Peaks smaller than 1% have been omitted.

[b]Repeated injections from the same sample solution.

[c]Injections from different sample preparations of the same oil.

TABLE 2

The Relative Representation of a Commercial Triglyceride Mixture Compared to the Nominal Value

Triglyceride	ECN-values[a]	Nominal value[b] (weight%)	Mass detection (area%) n = 3	UV-detection[c] at 215 nm (area%)
24:0	24.0	9.4	9.8	6.1
27:0	27.0	6.3	6.1	4.5
30:0	29.9	9.4	10.1	5.8
33:0	33.0	6.3	6.0	2.8
54:9	36.5	3.1	2.5	44.2
36:0	36.1	9.4	10.3	4.6
39:0	39.1	6.3	6.6	2.6
54:6	40.9	3.1	2.9	15.1
48:3	41.1	3.1	2.7	2.0
42:0	41.9	9.4	11.3	3.3
45:0	44.7	6.3	7.2	2.0
54:3	46.6	3.1	3.2	1.6
48:0	48.1	9.4	11.0	2.8
51:0	51.5	6.3	5.7	1.4
54:0	53.8	9.4	4.4	1.3

Linear gradient from 100% acetonitrile to 100% acetonitrile-ethanol-hexane (40:40:20, w/w/w).

[a]Calculated from the observed retention times in comparison to external standards.

[b]From Nu-Chek-Prep product bulletin regarding HPLC G-1 composition.

[c]The UV detector (Shimadzu SPD 2A) was connected in series with the mass detector.

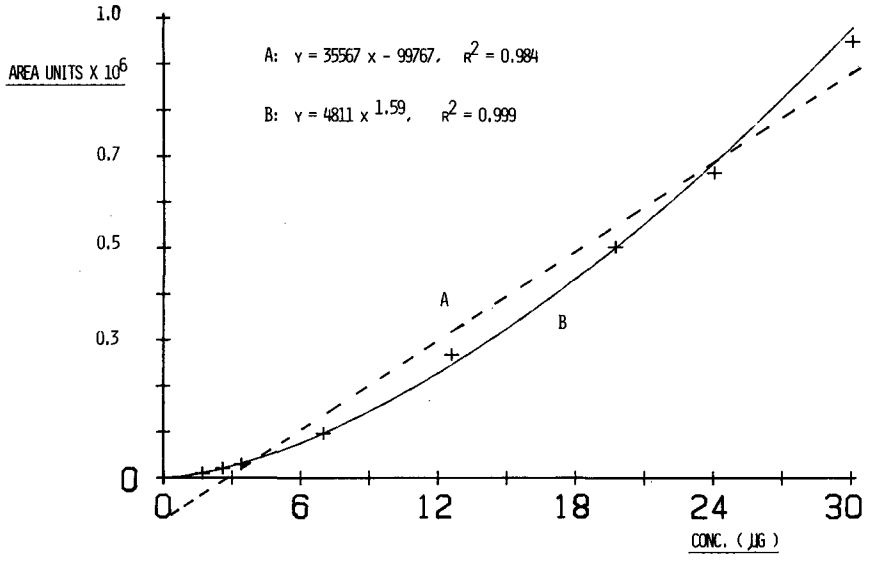

FIG. 5. Graphic representations of different amounts of trioctanoin against the response of the mass detector. The triglyceride amounts (range 0–30 µg) were plotted against the area units of the integrator. Linear regression (A) and a curve fit program (B) were applied on the same data points.

TABLE 3

Results from Treatment of Experimental Data Points of Triglyceride Standards by Linear Regression and Exponential Curve Fitting

Triglyceride	Linear regression ($y = ax + b$) a	b	r^2	Power curve ($y = ax^b$) a	b	r^2
27:0	3023	−48281	0.995	219.32	1.544	0.998
38:0	2504	−46704	0.985	51.78	1.819	0.994
40:0	2628	−54656	0.990	52.73	1.815	0.994
42:0	2528	−45489	0.987	39.77	1.879	0.994
45:0	2737	−58322	0.989	57.21	1.804	0.994
46:0	2467	−46590	0.985	69.02	1.745	0.998
48:0	2363	−37214	0.990	150.48	1.575	0.996
50:0	2610	−29403	0.995	195.47	1.559	0.998
51:0	2779	−34791	0.993	168.36	1.595	0.996

Injected amounts 20–100 µg.
r = correlation coefficient.

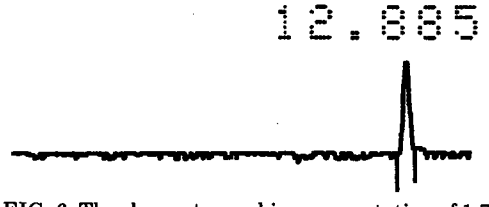

FIG. 6. The chromatographic representation of 1.7 µg trioctanoin by mass detection. The signal to noise ratio observed here gives a detection limit of approximately 1 µg of this triglyceride.

elucidation of triglyceride and other lipid mixtures in terms of reproducibility, quantitative possibilities and detection limits. In the general demand for universal detectors compatible to gradient elution needed by lipid chemists we believe this detector is an interesting and useful alternative.

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Quantitative Relationships Between Dietary Linoleate and Prostaglandin (Eicosanoid) Biosynthesis

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ABSTRACT

Essential fatty acid deficiency consistently depresses eicosanoid (prostaglandin E_2, F_2, and I_2 and thromboxane) biosynthesis independent of sampling protocols. Tissue fatty acid analyses support the hypothesis that the decrease is due in part to depression of arachidonate and accumulation of eicosatrienoate (n-9). Research on the alteration of eicosanoid biosynthesis by dietary linoleate supplementation is reviewed extensively. Responses of whole blood, lung, liver and heart eicosanoid synthesis to feeding eight concentrations of dietary linoleate between 0 and 27 energy percent are reported. It is concluded that stimulation, depression and no change in eicosanoid production could be equally well documented as a response to linoleate supplementation. Evidence for the obvious mechanism that alterations in precursor fatty acid composition are a possible explanation is fragmentary and inconsistent. The appropriate sampling techniques appear not to be established at this time and most likely are species, gender and tissue specific.
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INTRODUCTION

Eicosanoids are oxygenated unsaturated 20-carbon fatty acids that possess powerful and diverse pathophysiological actions. Eicosanoid is the presently preferred generic name because the classical prostaglandins (PG) form only a small portion of this group of compounds that has been expanded with the discovery of thromboxanes (TX), prostacyclin (PGI_2) and leukotrienes (LT). By far the major metabolic source of eicosanoid precursors is tissue stores of arachidonate (20:6 n-6; AA) necessary for the biosynthesis of the two series of PG, prostacyclin and TX and the four series of LT.

AA must be derived from dietary sources because the n-6 double bond is dietarily essential, but very little AA is consumed directly by humans. The requirement for n-6 polyunsaturated fatty acids is expressed as linoleate (18:2 n-6) and is based primarily on fatty acid composition of plasma lipids. It has been set between one and two energy percent (en%). We estimate that the average American diet (1) provides less than 0.2 en% AA and approximately 6 en% linoleate; thus, one can conclude that most of the tissue AA is derived from dietary linoleate. There is, however, renewed interest in occurrence and functional effects of essential fatty acid deficiency (EFD) in humans (2). The delineation of the roles that AA and linoleate play and the relative as well as absolute concentrations of these fatty acids needed to support essential membrane structure and eicosanoid biosynthesis also have received renewed interest.

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EFD consistently depresses eicosanoid production under various conditions (3–12) and probably accounts for reproductive, renal, inflammation and platelet aggregation failures but not dermal lesions of EFD (13). Prottey (14), Elias et al. (15), and Houtsmuller and Beek (16) concluded that even numbered chain length fatty acids with n-6,9 double bonds can support normal skin function without conversion to eicosanoids. Maintenance of membrane structure is the major quantitative function of linoleate, and support of eicosanoid production is quantitatively much less but one should not conclude that the latter is less important. Several authors have estimated from excretion of urinary metabolites that in the unstressed state the daily total body production of eicosanoids is no more than 1 mg and is derived primarily from PGE and F. This production quantitatively accounts for a minute portion of the approximately 10 g linoleate that is normally consumed. No estimate has been made of the excretion of eicosanoid metabolites via the feces, which probably is a larger amount (17).

The assessment of eicosanoid biosynthesis is severely compromised by a myriad of sampling techniques, sometimes inappropriate (5,11). The ideal method would be assessment of total body synthesis by determining urinary metabolites. This appears valid for PGE and F excretory products, but the metabolites found for PGI and TX are so numerous that researchers must be cautious. A similar problem exists for circulating metabolites, plus vast species differences and close association with the female reproductive cycle have been reported. Another limitation in measuring eicosanoid metabolite levels is that the specific organ or tissue source is unknown. A large portion of the urinary excretion of the primary eicosanoids is derived from renal synthesis and not renal clearance. An exception is the highly variable contribution of seminal fluid in man. The renal cell type that produces specific eicosanoids is critical for making physiological inferences and unfortunately is not well delineated. In order to assess specific tissue synthesis, first attempts involved homogenizing tissue in the presence of a cyclooxygenase inhibitor or an organic solvent for assessment of endogenous eicosanoid concentration; however, some synthesis during sampling is unavoidable. If one allows synthesis to occur from endogenous precursor pools without addition of cofactors, then ex vivo rates are assessed (5). An artifact that might be introduced is nonspecific release of free fatty acids by lipases that might not be activated under pathophysiological circumstances. A modification often used is addition of exogenous radiolabeled AA; the protocol is sensitive and easy to perform, but one should be cautioned that constant and uniform specific activity of the precursor pool(s) is essential for calculation of synthesis rates and often is not assured. A few investigators have elicited eicosanoid release from isolated cells or tissues or perfused organs by specific physiological challenges. Several levels should be employed, because a maximal challenge likely will evoke unphysiological eicosanoid concentrations. We emphasize the numerous precautions in sampling that must be taken in making proper inferences. Empirical observations along with theoretical considerations ultimately will provide valid protocols.

The purpose of this article is to review comprehensively the eicosanoid biosynthesis response to linoleate supplementation, defined as dietary linoleate concentrations greater than the essential fatty acid requirement. These responses could provide explanations for supplemental linoleate's beneficial and deleterious physiological actions, such as blood pressure regulation, thrombosis, lipolysis and inflammation. In addition, the evidence that the mechanism by which dietary linoleate alters eicosanoid production is via alteration of precursor pools will be discussed when the fatty acid profile analyses accompany the results. Only brief reviews are presently available (5,7,18). Tables 1, 2 and 3 summarize published results classified according to dietary linoleate content, species and tissue, sampling protocol and eicosanoid determined with no judgments placed on sampling processes or assay accuracy. Over half the reports found no statistically significant ($P > 0.05$) effect of supplemental dietary linoleate on blood (Table 1) or cardiovascular (Table 2) eicosanoid biosynthesis. Taken together, five articles reported a statistically significant ($P < .05$) increase and four a significant decrease with higher dietary linoleate. One is struck by the fact that excess dietary linoleate does not always cause enhanced eicosanoid production and that sampling and species, as well as eating pattern and gender (41; Taylor and Mathias, J. Am. Oil Chem. Soc. 60:723 [abstract #179], 1983), can alter the results. Blood is routinely sampled after an overnight fast, which was the case in all but one investigation reported in Table 1. We (41) have shown that fasting masks the effects of supplemental linoleate. The endogenous eicosanoid concentration of the other tissues studied—skin, lung, liver and kidney—showed a consistent enhancement independent of degree of linoleate supplementation (Table 3). When

TABLE 1

Effect of Supplemental Dietary Linoleate on Blood Eicosanoid Biosynthesis

Dietary fat, en%	Linoleate, en%		Gender, species and sample	Sampling procedures and challenges	Eicosanoid	Degree of response[a]	*Comments*	Ref. #
	Control	Supplementation						
38	1	24	Male rat blood	Clot at room temp	PGF_2, E_1	+41, +43%	Fasted and fed combined	19
40	3	25	Male rat washed platelets	Collagen	TX	ns	Assayed as MDA	8
40	4	up to 20	Male rat blood	Clot; 10 or 40 min	PGE_2, F_2, E_1	ns	20 en% fat also ns	20
25	2	12	Male rabbit PRP[b]	Collagen; 2 min	TX	ns		21
40	0.4	up to 29	Male rat blood	Clot; 10 or 40 min	PGE_2, F_2, E_1, TX	ns		22
25	5	9	Male rat blood	Clot; 30 min	TX	ns		23
25	5	9	Male rat plasma	Heparin; aspirin	TX	ns		23
*	6	17	Male rat washed platelets	Thrombin; 5 min	TX	−50%	*Total fat increased	24
50	3	32	Rat PRP	Collagen; 5 min	TX	ns	Assayed as MDA	12
50	3	32	Male rat PRP	Collagen; 5 min	TX	+92%	Assayed as HHT, response dependent upon collagen dose	12
40	8	up to 25	Male rabbit PRP	Collagen; 5 min	TX	ns		12
*	2	8	Male human PRP	Collagen; 2 min	TX	−17%	*Total fat decreased	25
*	2	8	Female human PRP	Collagen; 2 min	TX	+14%	*Total fat decreased	25
40	4	6	Male pig blood	Clot; 60 min	TX	ns		26

[a]ns, $p > 0.05$.

[b]PRP, platelet rich plasma.

TABLE 2

Effect of Supplemental Dietary Linoleate on Cardiovascular Eicosanoid Biosynthesis

Dietary fat, en%	Linoleate, en%		Species and tissue	Sampling procedures	Eicosanoid	Degree of response[a]	Comments	Ref. #
	Control	Supplementation						
40	5	28	Rabbit heart	Langendorff	PGI_2	ns	$KPGF_{1\alpha}$ GC analysis of pooled	27
35	4	25	Rat heart	Langendorff	PGI_2	ns	samples from rabbit and rat	27
35	4	25	Rat aorta	Perfused	PGI_2	ns	hearts showed decreases of	27
35	3	up to 25	Rat aorta	Pulsatingly perfused	PGI_2	+45%	−46 and −40% (average of two	8
35	3	25	Rat aorta	Pieces; 3 min	PGI_2	ns	experiments), respectively.	8
25	2	12	Rabbit aorta	Fragments; 20 min	PGI_2	−65%		21
40	7	22	Rat aorta	Endogenous	PGE_2, F_2	−44, −24%		28
36	4	25	Rabbit aorta	Pulsatingly perfused	PGI_2	ns		29
36	4	25	Rabbit aorta	Pieces; 4 min	PGI_2	ns		29
25	5	9	Rat aorta	Pieces; 15 min	PGI_2	ns		23
50	3	32	Rat aorta	Pieces; 4 min	PGI_2	ns		12
>40	6	22	Rat aorta	Rings; 5 min	$KPGF1_\alpha$	ns		30
40	4	6	Pig artery	Strips; 20 min	$KPGF1_\alpha$	+42%		26

[a]ns, $p > 0.05$.

tissue homogenates were incubated for varying lengths of time, the results became quite variable. Kidney and adipose tissue usually showed positive response to supplemental linoleate, whereas lung and mammary gland were negative. Urinary excretion is a sampling technique that possesses minimal artifact, and it consistently showed enhancement due to linoleate supplementation.

There is a paucity of data on dietary factors and LT synthesis. Theoretically, one should not expect LT production to respond to dietary linoleate in the same way as PG and TX production because (i) the substrate pool for cytosolic lipoxygenases will be quite different from the microsomal one for the cyclooxygenase, and (ii) the lipoxygenase substrate kinetics are quite different, 18 carbon polyunsaturated fatty acids are also hydroxylated and the n-6 double bond is not essential. Only one study on dietary linoleate has been reported. Nugteren et al. (43) investigated calcium ionophore-stimulated, carrageenin-elicited rat polymorphonuclear leukocytes, from which hydroxylated fatty acids were the major end products and not sulfido-peptide LT. EFD depressed production of 5-hydroxy-20:4, 94%; LTB_4, 76%; 9+13-hydroxy-18:2, 86%. 5-Hydroxy-20:3 but not LTB_3 appeared in the chromatogram from EFD leukocytes.

The existence of conflicting reports suggests that much more research needs to be conducted, and appropriate sampling methods are critical. In addition, most of the investigators cited in Tables 1, 2 and 3 fed only one level of supplemental linoleate. Our primary objective has been to expand the findings on response of eicosanoid biosynthesis to supplemental linoleate by feeding several concentrations in order to describe the quantitative response in greater detail. We investigated the effect of feeding eight (42) and five (41) concentrations of linoleate ranging from 0 to 29 en% for 6 and 3 mo, respectively, to female rats. Clotting whole blood was used for assessing rate of eicosanoid biosynthesis. The greatest dietary response was observed when the blood was collected in the fed state and allowed to clot for 10 min (Figs. 1 and 2). Rats fed 0 en% linoleate (fat-free diet) consistently showed depression, and ones fed 1 to 2 en% linoleate (fat source was primarily beef tallow) were consistently associated with the highest production of TX and PG. The increase in synthesis was associated with the disappearance of serum eicosatrienoic acid (20:3 n-9) and not a change in serum AA content (42, Fig. 3). In this regard, Parnham (6) did not find a correlation between the serum n-9/n-6 ratio and platelet derived PG

TABLE 3

Effect of Supplemental Dietary Linoleate on Tissue Eicosanoid Biosynthesis

Dietary fat, en%	Linoleate, en%		Species and tissues	Sampling procedures	Eicosanoid	Degree of response[a]	Comments	Ref. #
	Control	Supplementation						
38	4	23	Rat skin	Endogenous	PGF_2	+263%		31
20	1	up to 10	Rat adipocytes	Norepinephrine stimulated; 60 min	PGE_2, F_2	+358, +230%	40 en% similar response	32
40	2	31	Rat lung	Endogenous	PGE_2, F_2	+1200, +575%		33
40	4	up to 20	Rat lung	Endogenous	PGE_2, F_2	ns	20 en% similar response	5
40	4	up to 20	Rat lung	Homogenate; 10 min	PGE_2, F_2	−36, −34%	20 en% similar response	5
40	4	up to 20	Rat liver	Endogenous	PGE_2, F_2	+79, +93%	20 en% similar response	5
40	4	up to 20	Rat liver	Homogenate; 10 min	PGE_2, F_2	ns	20 en% similar response	5
29	4	20	Female human	Urine	M[b] PGE + F	+105%		34,35
40	0.6	up to 29	Male rat	Urine	M PGE + F	+188%	Calculated at peak response (2 wks); decreased with time	36
*	6	12	Rat kidney medulla	Homogenate; 10 min	PGE_2, F_2	ns	*Total fat increased	37
38	4	25	Rat mammary gland	Homogenate	PGE_2, F_2	ns, −72%		38
40	7	22	Rat kidney	Endogenous	PGE_2	+155%		28
40	2	27	Rat kidney cortex	Homogenate; 60 min	PGE_2, TX	+45%, ns		39
40	2	27	Rat kidney medulla	Homogenate; 60 min	PGE_2, TX	ns, +75%		39
40	0.4	up to 29	Male rat	Urine	PGE_2, F_2, E_1, TX, $KPGF_{1\alpha}$	ns, ns, ns, 82%, ns		22
20	0.2	up to 11	Male rat	Urine	PGE_2, F_2, E_1 TX, $KPGF_{1\alpha}$	+96, ns, +253, +265, +123%		22
38	3	30	Rat kidney papilla	Endogenous	PGE_2	+158%		40

[a]ns, $p > 0.05$.

[b]M, metabolite of.

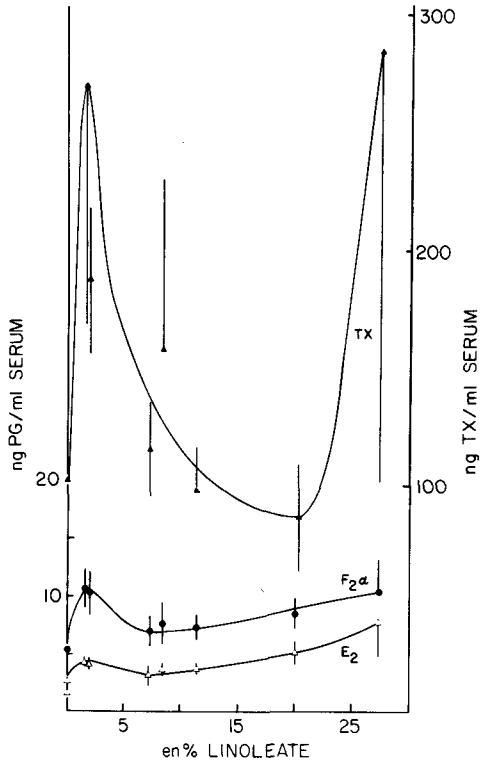

FIG. 1. Effect of dietary linoleate on eicosanoid synthesis during clotting of whole rat blood at 37 C for 10 min. Female rats were fed the diets for 6 mo and fed a meal before killing. Means and SE (n = 9) are plotted (42).

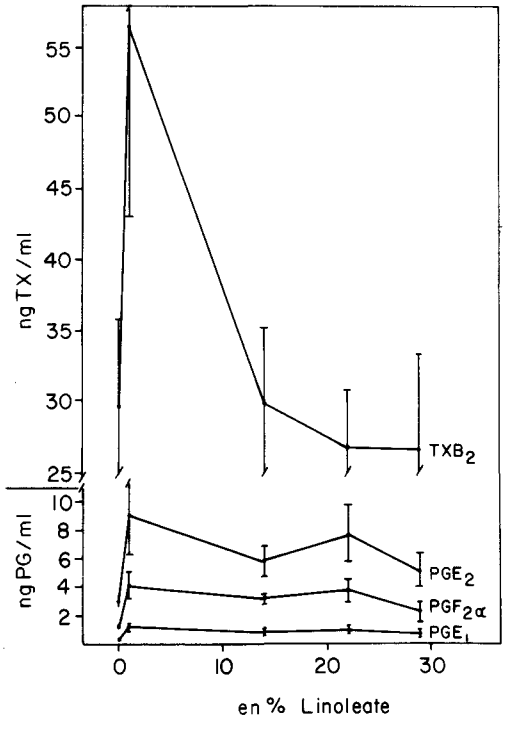

FIG. 2. Effect of dietary linoleate on eicosanoid synthesis during clotting of whole rat blood at 37 C for 10 min. Female rats were fed the diets for 13 wk and fed a meal before killing. Means and SE (n = 9) are plotted (41).

synthesis. A more specific assessment that the effect of EFD on eicosanoid production is due to changes in precursor fatty acid composition is to determine the composition of membrane phospholipid fatty acids. Hornstra et al. (12) and Hwang et al. (44) reported that EFD induced by feeding hydrogenated coconut oil decreases platelet phospholipid AA which is closely associated with depressed TX production.

Between 2 and 10 en% linoleate, a trend for reduced eicosanoid synthesis was observed and was associated with a decline in serum phospholipid AA (Fig. 3). Between 10 and 20 en% linoleate, TX synthesis, which was the major eicosanoid released, had returned to EFD rates. When safflower oil (providing about 28 en% linoleate) was fed, the response was not the same in the two experiments, enhancement (42, Fig. 1) and no effect (41, Fig. 2) relative to the 20 en% rates were observed. The lack of an effect in the second experiment might be due to the shorter feeding time. The alterations in

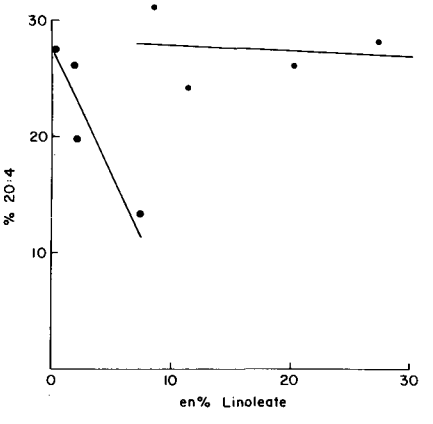

FIG. 3. Effect of dietary linoleate on proportion of arachidonate (20:4 n-6) in rat serum phospholipids (42).

eicosanoid synthesis observed between 10 and 28 en% linoleate were not closely associated with changes in serum phospholipid AA content. A similar lack of association between precursor concentration and eicosanoid production has been reported (12,44). Galli et al. (21) reported that supplemental linoleate replaces AA in rabbit platelet phospholipids; however, they could find no significant depression in TX synthesis by collagen-challenged platelet-rich plasma (PRP). We conclude from these studies that response of platelet TX synthesis to dietary linoleate supplementation is not simply linear. In general, the response is negative when sampling is performed during the fed state, and it has been difficult to show that alteration in eicosanoid synthesis is due to changes in precursor fatty acid profiles.

Liver, lung and heart also were sampled in the former study (42) to assess the endogenous content and ex vivo biosynthesis of eicosanoids. Several approaches have been used to assess endogenous eicosanoid content; we chose homogenizing tissues in a high concentration of aspirin, an irreversible cyclooxygenase inhibitor, as the most rapid system that is compatible with radioimmunoassay (RIA) analysis. There is certainly some eicosanoid production during the handling of the organ, but we see little advantage to rapid freezing then extraction with organic solvents during thawing. The time courses for ex vivo biosynthesis have been published (5,45,46). This model is useful for nutrition studies in which the alteration of endogenous substrate pools is the subject under investigation.

The predominant eicosanoid produced by rat lung and heart is PGI_2, and the kinetic properties of PGI_2 synthetase might be different from the TX synthetase found in platelets. Most researchers (Table 2) have determined PGI_2 by bioassay taking advantage of its powerful antiaggregatory properties. Unfortunately, the assay must be performed immediately because PGI_2 spontaneously decomposes to 6-keto-$PGF_{1\alpha}$ ($6KPGF_{1\alpha}$). We took advantage of the latter property and developed a specific RIA for $6KPGF_{1\alpha}$ which could be used with unextracted tissue homogenates.

EXPERIMENTAL PROCEDURES

The details of animal care and diets have been published (42). Briefly, female pups from dams fed varying but adequate concentrations of linoleate were fed one of eight concentrations of dietary linoleate for six mo (n = 9). The composition of the 40 en% fat basal diet included (on a weight basis) 21.2% fat, 19.0% lactalbumin, 50.3% carbohydrates, 2.5% cellulose, 5.0% salt mix and 2.0% vitamin mix. EFD (0 en% linoleate) was induced by feeding a fat-free diet. Mixtures of beef tallow, soybean oil and safflower oil provided 1.7, 2.0, 7.4, 8.5, 11.3, 20.2 or 27.4 en% linoleate by actual analysis. The rats were fasted overnight; then, two hr after a one-hour meal, blood was drawn under ether anesthesia. Portions of liver, lung and heart were then rapidly removed, weighed and homogenized in 10 volumes of ice-cold potassium phosphate buffer, pH 7.4, (KPi) using a Teflon pestle and glass vessel (Arthur Thomas, Philadelphia, Pennsylvania). To estimate endogenous eicosanoid content, the KPi contained 42 mM aspirin. To estimate ex vivo eicosanoid biosynthesis, the homogenates were incubated for 10 min (liver and lung) or 60 min (heart) in a gyro water bath shaker at 37 C. Eicosanoid biosynthesis was stopped by adding aspirin at a final concentration of 14 mM. Samples were stored frozen until eicosanoids were determined by RIA.

PGI_2 and TXA_2 concentrations were measured as their stable spontaneous decomposition products, $6KPGF_{1\alpha}$ and TXB_2, respectively. $PGF_{2\alpha}$ and PGE_2 were directly determined by double-antibody (Method 2) RIA as previously described (46). All assays were done under equilibrium conditions in a phosphate-buffered, pH 7.0, saline (PBS) solution containing 0.1% gelatin at 4 C. Unextracted samples were incubated in the presence of tritiated eicosanoid (New England Nuclear, Boston, Massachusetts) and eicosanoid-specific (19,22) rabbit anti-serum (in PBS containing .01M EDTA which had been preincubated with sheep anti-rabbit gamma globulin) at a final volume of 1.0 ml for 24 hr. Following centrifugation, unbound constituents were decanted and the sedimented radioactive bound fraction was counted using liquid scintilliation techniques. Logit transformation, curve fitting and averaging were performed by the computer program described by Duddleson et al. (47). The interassay variability established using quality control pools is large but not unusual: $PGF_{2\alpha}$, 9%CV; PGE_2, 24%CV; TXB_2, 9%CV and $6KPGF_{1\alpha}$, 6%CV (n = 17). To control for this contribution to variability the samples were arranged so that each treatment was represented equally in each assay.

The incubated tissue samples and endogenous lung $6KPGF_{1\alpha}$ were found upon dilution to be parallel to the standard curve (47). The endogenous liver and lung contents bound between 50 and 80%; thus, parallelism could not be demonstrated with certainty. No nonspecific binding was observed in these samples, and

recovery of non-labeled standards was greater than 90%.

One-way analyses of variance (OWAV) were calculated using a computer program (Hewlett-Packard, Loveland, Colorado), and the pooled SE calculated from the error mean squares are reported as indicative of variance.

RESULTS

Of the determined eicosanoids, the major cyclooxygenase product was $PGF_{2\alpha}$ in liver (Figs. 4 and 5) and $6KPGF_{1\alpha}$ in lung (Figs. 6 and 7) and heart (Fig. 8), and the liver and lung eicosanoid concentrations increased 20- to

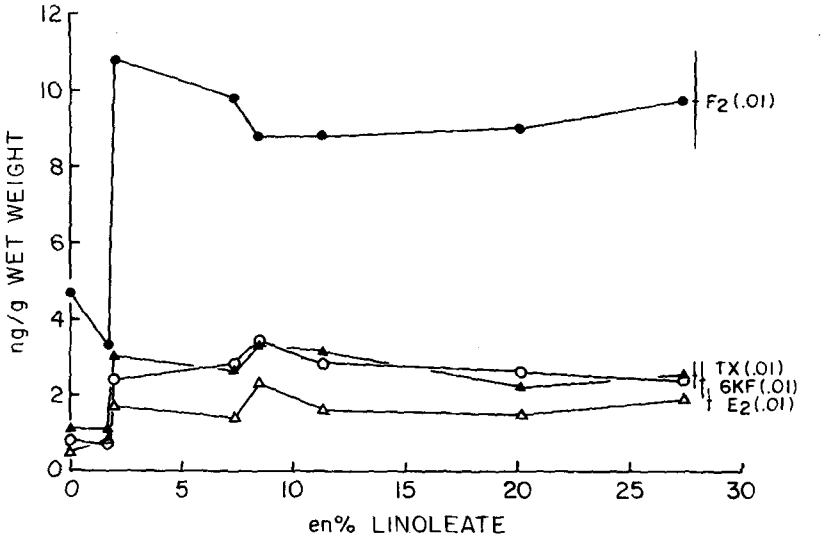

FIG. 4. Effect of dietary linoleate on endogenous hepatic eicosanoid content from female rats fed diets for 6 mo. The abbreviations for designating eicosanoids are F_2 for $PGF_{2\alpha}$, E_2 for PGE_2, TX for TXB_2, and 6KF for 6-keto-$PGF_{1\alpha}$. Probability level from OWAV shown in parentheses, and bar indicates pooled SE.

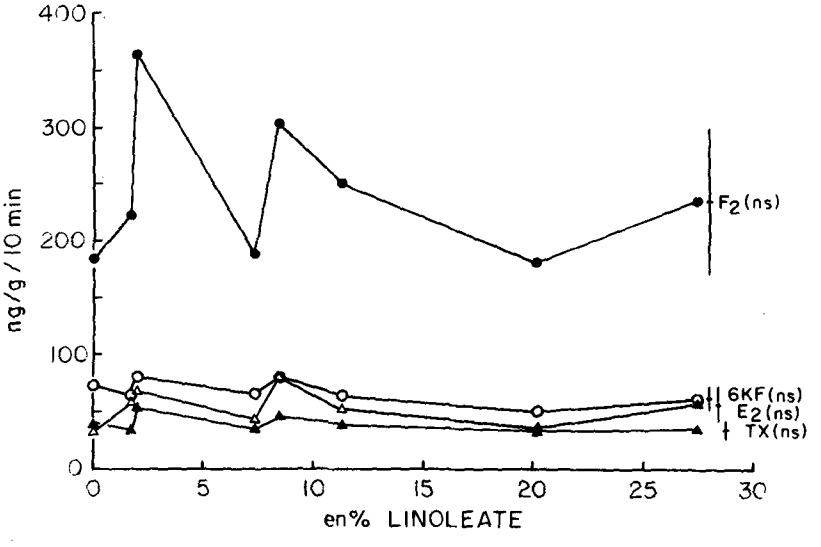

FIG. 5. Effect of dietary linoleate on hepatic eicosanoid synthesis from female rats fed diets for 6 mo. Probability level from OWAV shown in parentheses, and bar indicates pooled SE.

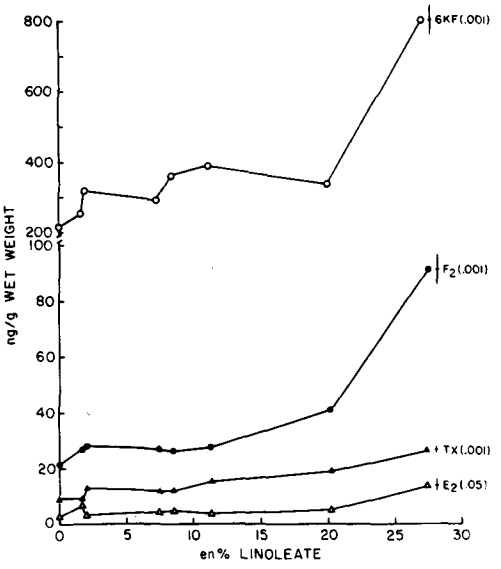

FIG. 6. Effect of dietary linoleate on endogenous pulmonary eicosanoid content from female rats fed diets for 6 mo. Probability level from OWAV shown in parentheses, and bar indicates pooled SE.

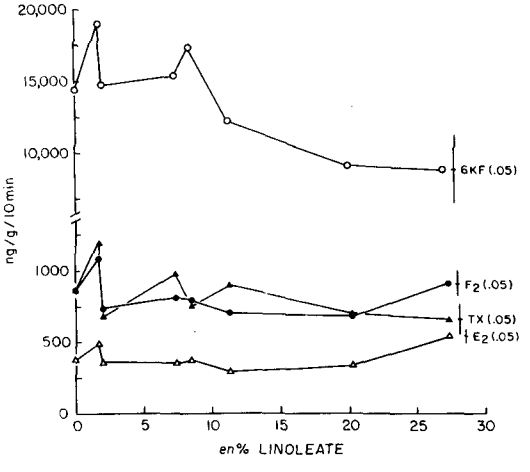

FIG. 7. Effect of dietary linoleate on pulmonary eicosanoid synthesis from female rats fed diets for 6 mo. Probability level from OWAV shown in parentheses, and bar indicates pooled SE.

50-fold during the 10-min incubation.

Consumption of 0 or 1.7 en% linoleate depressed by about 50% ($P < .01$) the endogenous hepatic eicosanoid content when compared to 2 to 27 en% linoleate (Fig. 4). This depression was associated closely with the AA content of total phospholipids isolated by thin

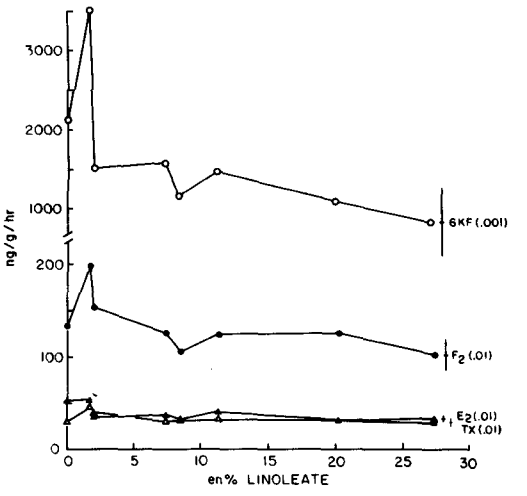

FIG. 8. Effect of dietary linoleate on cardiac eicosanoid synthesis from female rats fed diets for 6 mo. Probability level from OWAV shown in parentheses, and bar indicates pooled SE.

layer chromatography (TLC). The former's content was 13%, whereas the latter's was 23% (data not shown). The dietary linoleate effect was abolished if the liver homogenates were incubated ex vivo (Fig. 5). The endogenous pulmonary eicosanoid content (Fig. 6) increased linearly as the dietary linoleate was increased ($r = .83$, $6KPGF_{1\alpha}$; .86, $PGF_{2\alpha}$; .96, TXB_2 and .72, PGE_2). An opposite—that is negative—linear response was observed when the lung homogenates were incubated (Fig. 7, for example, $r = -.82$, $6KPGF_{1\alpha}$). A nearly identical decrease is reported for cardiac $6KPGF_{1\alpha}$ synthesis (Fig. 8). Unfortunately, pulmonary and cardiac fatty acids were not determined.

DISCUSSION

EFD depressed the endogenous hepatic and pulmonary eicosanoid contents, which is consistent with reports in the literature. The mechanism is presumed to be a lack of precursor fatty acid, AA. As discussed earlier, this is the case in platelets (6,12,44) and is what we observed in the liver; however, Willis et al. (10) reported a marked decrease in endogenous PGE_2 and $PGF_{2\alpha}$ content in several rabbit tissues but no depression in liver, erythrocyte or brain phospholipid AA. It is of utmost importance to realize that crude analyses of fatty acid composition do not reflect the true eicosanoid precursor pool and that composition of dietary fat cannot be assumed to result in linear

changes in tissue linoleate and its products. Other mechanisms, such as specificity and activity of lipases (48), inadequate peroxide tone due to lack of PGG synthesis (49), or compensatory induction of cyclooxygenase enzyme synthesis (50-52), also might explain this anomalous effect of EFD.

The effect of linoleate supplementation on eicosanoid synthesis is not consistent. For the tissues described herein, other reports have appeared showing no effect, an enhancement or a decrease (Table 3). We also observed all three responses in relation to dosage and duration of supplementation. Lands (48) and Galli et al. (53,54) have pointed out that control of precursor fatty acid pools could occur at several loci: conversion of linoleate to AA, deposition and turnover of long chain polyunsaturates, specific vs. general release of AA, competition between cyclo- and lipoxygenase, and kinetic properties of eicosanoid synthetases. If unphysiological nonspecific release of fatty acids did not occur and if indeed the depression of eicosanoid production caused by dietary linoleate is the more correct observation, two obvious mechanisms exist. It is possible that linoleate may act to displace AA at the tissue level (21), thereby inhibiting availability of the necessary eicosanoid precursor. Also, high tissue linoleate may act as a competitive inhibitor of AA for cyclooxygenase (55,56), successfully causing a depression of eicosanoid synthesis. In addition, one cannot rule out aspects of other dietary factors such as vitamin E and cholesterol.

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Non-Soluble Dietary Fiber Effects on Lipid Absorption and Blood Serum Lipid Patterns

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ABSTRACT

Generalized effects of dietary fiber on lipid absorption and blood serum lipid patterns of humans have not been defined and may not even exist. The term dietary fiber covers a wide variety of materials with different chemical and physical characteristics. The ability of pectins and mucilages, often classed as soluble fibers, to lower blood and liver lipids has been demonstrated repeatedly and consistently. However, demonstrated hypolipidemic effects of feeding such non-soluble fibers as cellulose, hemicellulose and bran are by no means consistent. On the basis of pooled data, it appears that hypolipidemic response or non-response of humans to inclusion of non-soluble fibers in diets is in part related to the degree of fecal bulking as a result of in vitro water holding capacity and in part related to pre-study blood serum lipid levels of the individual subjects.
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Among the postulated benefits of increasing the fiber content of the typical American diet is the assumed reduction both in blood serum lipids and in occurrence of atherosclerosis and coronary heart disease. Part of the evidence used to support this hypothesis has been collected from epidemiological surveys and observations (1-6). However, cause and effect relationships are always difficult to ascertain in studies of this type because many other dietary and non-dietary variations may be present.

Relationships among dietary fiber intake, fat absorption and blood serum lipids as demonstrated in controlled studies with animals and humans are by no means clear-cut. Several excellent reviews of the current state of knowledge regarding fiber and lipid absorption and metabolism have been published recently (7-12).

Part of the problem in defining effects of edible fiber on lipid metabolism is that of definition of the term edible fiber, because the term covers a large number of materials with widely different chemical and physical properties (7,8). Most, but not all, are plant cell wall components and many are also complex carbohydrates. The single most consistent characteristic of an edible fiber is that it is a food or feed constituent which is relatively resistant to digestion by enzymes found normally in the upper mammalian intestinal tract. Other authors have used different terms to designate this material. Considering the wide range of materials included in this group, it is not surprising that dietary fibers might produce different effects in controlled intervention studies designed to study fiber-lipid interactions, or that more than one mechanism might be involved in the elucidation of similar effects by different fiber sources.

Edible fibers often are subdivided on the basis of relative apparent solubility in water. Pectins, gums, mucilages and some hemicelluloses are classed together as water-soluble fibers or viscous polysaccharides. Actually, these substances do not form true solutions because of their large molecular size; however, they are capable of forming gels. Insoluble fibers include celluloses, lignins and many hemicelluloses.

Several animal and human feeding studies indicate that the inclusion of many kinds of soluble fiber results in a lowering of blood cholesterol and/or triglyceride levels (13-23). However, results of studies involving non-soluble fibers and effects on blood serum lipids have been much less consistent (7,13,24-43). Pectin and guar gum (soluble fibers) and cellulose and hemicellulose (insoluble fibers) have been investigated most extensively. Of non-purified sources, effects of wheat bran have been studied most often.

The ability of some dietary fibers (pectins, lignins, mucilages) to directly bind considerable amounts of bile acids and cholesterol and thus lower the reabsorption of endogenous cholesterol and cholesterol metabolites as well as the absorption of exogenous cholesterol has been suggested as the hypocholesterolemic mechanism of feeding some but not all dietary fibers (7,44-46). Pectin, mucilaginous fibers and lignin have been credited with possessing this physiochemical property and have been found to have hypocholesterolemic effects in many in vivo and in vitro studies. Guar gum, a neutral compound, is as effective a hypocholesterolemic agent as is pectin; hence, binding of bile acid evidently is not essential for a fiber to lower blood cholesterol levels. It may be that the cholesterol lowering effect of these substances is related to the sequestering of bile acids based

on gel formation. Although pectin is completely digested in the colon, it is doubtful that bile acids can be absorbed at this point in the lower gastrointestinal tract.

Anderson and Chen (7) proposed three mechanisms by which various plant fibers may influence blood serum cholesterol levels. These include altering intestinal absorption, metabolism and release of cholesterol, altering hepatic metabolism and release of cholesterol or altering peripheral metabolism of lipoproteins. In addition to the binding of bile acids and cholesterol by certain fibers, formation of gels by some fibers may reduce intestinal absorption of partition of the cholesterol. Dietary fiber may also alter bile acid metabolism in the large intestine by changes in microflora.

Results of several studies indicate that at least some kinds of dietary fiber may influence the production, metabolism and/or release of cholesterol from the liver and pancreatic activity (47-51). In addition, some kinds of dietary pectin have been found apparently to aid in lowering blood serum triglyceride levels with greater effects apparently occurring in postprandial than in fasting state subjects (18,52,53). As with cholesterol lowering effects of dietary fiber, triglyceride lowering effects of dietary fiber may be a result of alterations in intestinal handling of fats, hepatic metabolism of fatty acids and triglycerides and/or metabolism of lipoprotein. Increased fecal fat loss concurrent with the feeding of some kinds of dietary fiber have been observed. Fecal fat absorption may be slowed and occur at lower points in the intestinal tract when dietary fiber is increased.

Many studies have sought to establish relationships between increased intakes of certain kinds of dietary fiber and reduced pancreatic insulin release. This might result in reduced stimulation of fatty acid triglyceride synthesis (15,53,54).

Research on effects of dietary fiber on high density lipoprotein and phospholipid levels is rather limited. In general, it appears that these fractions are little affected by changes in dietary fat intake. Cellulose and hemicellulose are among the most commonly consumed forms of dietary fibers. Cereal brans are the major contributors of these fiber types to human diets. Not only do corn, wheat, rice and oat brans differ in their dietary fiber composition, but considerable variation in fiber composition among wheat brans from various sources also has been reported, making comparison of results from different laboratories difficult. Oat bran, which in general contains higher levels of soluble fibers than do other brans, has been reported to have hypocholesterolemic and hypotriglyceridemic effects (40,46,56-59). The fiber content of wheat bran, for the most part, is composed largely of insoluble fiber. The hypocholesterolemic and hypotriglyceridemic effect of wheat bran is controversial (60-69).

In this laboratory, even when insoluble fibers are used as the dietary fiber source in human feeding studies, blood serum lipids of some subjects appear to be affected while those of other subjects appear to be unaffected. Part of this seeming discrepancy may be due to experimental errors. However, it may be that "responders" have characteristics which, when interacted with dietary fiber, result in a lowering of blood lipid levels. If so, identification of these characteristics and factors is warranted.

The objective of the current research was to determine factors influencing response and nonresponse of human subjects to inclusion of moderately high levels of insoluble dietary fiber on serum lipid patterns of normolipidemic human adults.

PROCEDURES

Data used in this project were drawn from a series of 28 individual studies ranging in length from 21 to 56 days, with experimental periods ranging from 7 to 14 days in length. In studies involving 14-day periods, no differences were found between responses following the first seven days and those following the second seven days. Hence, seven-day periods were judged of sufficient length. The studies involved eight to 12 apparently healthy adult human subjects each. A total of 285 human subjects, primarily University of Nebraska students, participated in them. Subjects ranged in age from 19 to 40 yr (mean, 26.8 yr) with weight proportional to height. A total of 248 women and 37 men participated. Individuals were approved for participation by medical personnel of the University of Nebraska Health Center. Individuals with initial blood serum cholesterol levels of greater than 220 mg/dl and/or triglyceride levels of greater than 120 mg/dl were not included in this project. The project was approved by the University of Nebraska Institutional Review Committee for Studies Involving Human Subjects. All subjects signed subject consent forms prior to the start of the study.

All subjects were fed laboratory controlled, measured diets composed of ordinary foods. Foods included skim milk, peanut butter, bread, peaches, pears, green beans, stewed tomatoes, orange juice, tuna fish, lean ground beef, rice, macaroni, margarine, soft drinks, jelly and hard

candy. Caloric intake for any one subject was kept constant but was varied among subjects for weight maintenance. This diet provided approximately 30% of the energy from fat and less than 100 mg of cholesterol per subject per day. Subjects maintained usual activity patterns except for eating the controlled diet under supervision of the departmental metabolic unit, for making complete collections of urine and stools, and for giving fasting venous blood samples.

Experimental diets were fed with and without test dietary fibers. Each subject received both diets. The order of experimental periods was randomized in a cross-over design. Test dietary fibers included psyllium seed fiber (98 subjects), cellulose (30 subjects), rice bran (10 subjects), corn bran (10 subjects) and wheat bran (137 subjects). Because the brans contained less fiber than did the purified fiber sources, it was necessary to feed more of these to equalize actual fiber intake at 16 g per day. The two wheat brans were obtained from the American Association of Cereal Chemists which has obtained, analyzed and stockpiled these two brans specifically for research purposes. Fiber analyses for AACC certified soft white and hard red wheat brans as supplied by the American Association of Cereal Chemists were as follows: crude fiber, 10.20% and 10.30%; acid detergent fiber, 11.10% and 13.70%; neutral detergent fiber, 38.30% and 43.20%; lignin, 2.80% and 4.10%, and pectin, 16.78% and 11.00%. Test fibers were fed as an ingredient in a bread product or as a peanut butter-fiber mixture. These fiber-enriched foods or the same foods without fiber enrichment were fed in equal amounts at each of the three daily meals as determined by the experimental design.

Fasting venous blood samples were drawn and analyzed for serum total cholesterol and triglyceride contents using an Auto Analyzer II (Technicon) and HDL-cholesterol by heparin-manganese precipitation (70). Blood serum phospholipid content also was measured (71-73). Subjects made complete collections of feces throughout all studies. These were divided and composited into period lots representing food eaten for each seven-day experimental period by use of fecal dye (carmine red) and bead markers. Fecal transit time was estimated by use of fecal dye (brilliant blue) and bead markers. Total wet fecal weight, dry fecal weight and fecal fat excretions (74) were determined.

All data from all studies were grouped for purposes of statistical analyses. Each subject was classed as a "responder" or a "non-responder" on the basis of effect of addition of dietary fiber on serum lipid levels. An individual with a decrease of 5% or more in serum cholesterol or triglyceride levels with fiber addition was classed as a responder. Others with smaller, no or opposite effects were classed as non-responders. These classes were then used in calculating other response parameters characterizing these groups. Data were subjected to statistical analyses including analysis of variance and Duncan's Multiple Range Test, using services of the University of Nebraska Biometrics Laboratory with advice from statisticians from that laboratory.

RESULTS AND DISCUSSION

Comparison of blood serum cholesterol levels of responders and non-responders with dietary fiber additions are given in Table 1. As would be expected given the classification system used, responders showed statistically significant decreases in blood serum cholesterol levels with fiber additions while non-responders did not. However, responders tended to have higher blood serum cholesterol levels than did the non-responders when the non-fiber-enriched diets were fed ($P < 0.0732$). These results would suggest that many of the non-responders simply already had achieved minimum blood cholesterol levels, and further decreases in blood cholesterol levels were not possible. However, the cholesterol levels of the non-responders tended to be higher than were those of the responder group following fiber addition ($P < 0.0916$).

Several other characteristics of responders in comparison to non-responders to fiber on blood serum cholesterol levels were found. The mean fecal transit time of the responders tended to be longer when fed the non-fiber-supplemented diets than was that of the non-responders. However, addition of dietary fiber resulted in a significant lowering of fecal transit time of the responders but not of the non-responders. However, addition of dietary fiber resulted in a significant lowering of fecal transit time of the responders but not of the non-responders. Wet fecal weights of responders increased significantly when fiber was fed, but this was not true of the non-responders. Two implications of these results are that many of the non-responders already exhibited good gastrointestinal tract motility and addition of dietary fiber had little or no effect on fecal transit time, or that the degree of fecal bulk increase due to water holding ability was less for the particular fibers used for the non-responders than for the responders. It would appear that the fecal bulking characteristics of the non-absorbable fibers

TABLE 1

Characteristics of Individuals Who Responded to Cholesterol Lowering Effects of Non-Soluble Dietary Fibers

	Non-Responders* (N = 120)		Responders* (N = 165)	
Characteristic	− Fiber	+ Fiber	− Fiber	+ Fiber
Blood serum cholesterol (mg/dl)	$172^{ab} \pm 15$	$172^{ab} \pm 16$	$186^{a} \pm 21$	$164^{b} \pm 19$
Blood serum HDL cholesterol (mg/dl)	$57^{a} \pm 6$	$56^{a} \pm 8$	$58^{a} \pm 7$	$53^{a} \pm 6$
Blood serum triglyceride (mg/dl)	$97^{ab} \pm 12$	$93^{ab} \pm 7$	$105^{a} \pm 11$	$90^{b} \pm 9$
Blood serum phospholipid (mg/dl)	$219^{a} \pm 25$	$201^{a} \pm 20$	$223^{a} \pm 30$	$210^{a} \pm 19$
Wet fecal weight (g/day)	$77.0^{a} \pm 10$	$99.8^{ab} \pm 20$	$65.2^{a} \pm 8$	$128.3^{b} \pm 11$
Dry fecal weight (g/day)	$19.2^{a} \pm 3$	$28.8^{b} \pm 5$	$20.8^{a} \pm 4$	$32.3^{b} \pm 6$
Fecal transit time (hr)	$42^{a} \pm 12$	$36^{ab} \pm 15$	$48^{a} \pm 16$	$30^{b} \pm 12$
Fecal fat (%)	$1.43^{a} \pm 0.18$	$1.62^{ab} \pm 0.12$	$1.23^{a} \pm 0.16$	$1.98^{b} \pm 0.18$

*Mean values of responder and non-responder subjects when fed diets with and without added non-soluble fiber supplement.

**Values with different letter superscripts were significantly different from one another ($P < 0.05$).

TABLE 2

Characteristics of Individuals Who Responded to Triglyceride Lowering Effects of Non-Soluble Dietary Fibers

	Non-Responders* (N = 135)		Responders* (N = 150)	
Characteristic	− Fiber	+ Fiber	− Fiber	+ Fiber
Blood serum triglyceride (mg/dl)	$99^{a**} \pm 16$	$101^{a} \pm 19$	$104^{a} \pm 20$	$83^{b} \pm 10$
Blood serum cholesterol (mg/dl)	$178^{ab} \pm 18$	$174^{ab} \pm 15$	$181^{a} \pm 19$	$166^{b} \pm 10$
Blood serum HDL cholesterol (mg/dl)	$57^{a} \pm 6$	$56^{a} \pm 8$	$57^{a} \pm 7$	$54^{a} \pm 8$
Blood serum phospholipid (mg/dl)	$218^{a} \pm 23$	$202^{a} \pm 21$	$224^{a} \pm 29$	$209^{a} \pm 21$
Wet fecal weights (g/day)	$78.1^{a} \pm 9$	$101.1^{ab} \pm 19$	$63.9^{a} \pm 7$	$129.0^{b} \pm 12$
Dry fecal weights (g/day)	$20.0^{a} \pm 4$	$28.9^{ab} \pm 6$	$20.3^{a} \pm 4$	$32.5^{b} \pm 6$
Fecal transit time (hr)	$42^{a} \pm 12$	$35^{ab} \pm 15$	$43^{a} \pm 15$	$30^{a} \pm 12$
Fecal fat (%)	$1.45^{a} \pm 17$	$1.60^{ab} \pm 0.13$	$1.20^{a} \pm 0.15$	$2.01^{b} \pm 0.19$

*Mean values of responder and non-responder subjects when fed diets with and without added non-soluble fiber supplement.

**Values with different letter superscripts were significantly different from one another ($P < 0.05$).

were important in promoting a lowering of absorption of dietary cholesterol.

Fecal fat excretion also tended to be higher with increase in dietary non-soluble fiber for both the responder and non-responder group. However, the degree of increase in excretion was greater for the responder than for the non-responder group. In an earlier study, fecal bulking effects of several forms of dietary fiber were not found to be related to hypocholesterolemic effect (75). However, in that study comparative effects involved very limited numbers of subjects.

Recalculation of data into responder and non-responder groups on the basis of triglyceride level changes are shown on Table 2. Fewer subjects in this project responded with a decrease in fasting level blood serum triglyceride levels with dietary supplements than with a decrease in blood serum cholesterol levels. It may be that more pronounced changes would have been observed closer to meal consumption times when triglyceride levels would have been expected to reach peak levels. In addition, the relatively low fat content (30% of energy) and high carbohydrate content (58% of energy) may have promoted increased rather than decreased levels of blood serum triglyceride levels.

Responders to hypotriglyceridemia effects of fiber tended to show somewhat directionally similar characteristics in comparison to characteristics of responders to hypocholesterolemic effects of non-soluble fiber. Increase in wet fecal weight, increase in water content of stools, decrease in fecal transit time and increase in fecal fat excretion were greater for responders than for non-responders.

The results of this study indicate some differences among responders and non-responders to the hypolipidemic effect of non-absorbable fibers. Usual gastrointestinal tract function and blood serum lipid levels may have been subject characteristics contributing to their response or nonresponse. However, the water holding ability of the non-soluble fibers also was important.

Mean effects of the test fibers used on the parameters in the comparisons of responders and non-responders were reported in earlier studies from this laboratory (39,63-67). Psyllium seed fiber, rich in hemicellulose, had the greatest water holding ability, fecal bulking ability, greatest influence on reducing fecal transit time, greatest influence on fecal fat loss and the greatest influence on reducing blood serum cholesterol and triglyceride levels. Red wheat bran, followed by white wheat bran and rice bran, also showed similar directional influences on these parameters but to a progressively lesser degree. Cellulose and corn bran, which had little water holding ability, had no demonstrated hypolipidemic effects when fed to human subjects.

These results would suggest that definition of water holding ability of a non-absorbable fiber source would be a good predictor of the in vitro hypolipidemic response of humans. However, in vivo methods of measuring water holding ability of dietary fibers do not give similar results (76). Fibers behave differently in different food systems, and in vitro and in vivo measurements are subject to differences. Thus, to say measuring water holding ability and bulking characteristics of non-soluble dietary fiber will give exact predictions of hypolipidemic influence is an over-simplification.

In conclusion, it would appear from results of this study that hypolipidemic effects of consuming non-soluble dietary fibers are in part related to characteristics of the individuals consuming the fiber and in part to the characteristics of the fibers themselves.

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Effect of Moderate to Very Low Fat Defined Formula Diets on Serum Lipids in Healthy Subjects

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ABSTRACT

Serum total cholesterol, high density lipoprotein (HDL) cholesterol, and triglyceride levels were studied in healthy male and female subjects consuming for one-week periods a diet of conventional food (CF) providing 42% of energy as fat, principally butter fat, and then in random order nutritionally complete, defined formula diets of moderate (32%) to very low (1%) fat content. Compared to CF, the formula with 32% of energy as corn oil lowered serum cholesterol by 25% and the ratio of total to HDL-cholesterol by 13%. Low (9%) and very low (1–3%) fat formulas reduced HDL-cholesterol by as much as 40%, raised the total:HDL-cholesterol ratio by about 20% and raised serum triglyceride levels by as much as 100%. When low and very low fat formulas were ingested for three weeks, these effects persisted although maximal responses occurred during the first week. These results demonstrated that a moderate fat formula diet with a high P/S ratio had a more favorable effect on serum lipid levels than various low fat formulas. Low fat conventional food diets should be studied in long-term controlled metabolic experiments before such diets are recommended to the general population for coronary heart disease or cancer prevention.
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INTRODUCTION

The positive association of low density lipoprotein (LDL) cholesterol with coronary heart disease (CHD) and the inverse relationship of high density lipoprotein (HDL) cholesterol with CHD are well known (1–3). Although elevated serum triglycerides are common in patients with CHD, some researchers have proposed that the relationship is not causal (4–7). High serum triglycerides are associated with other known risk factors, namely reduced HDL-cholesterol concentrations (5).

Dietary modulation of serum lipids is controversial and has attracted considerable research interest for several decades. There is evidence that reduced and modified fat intakes (to 30% of calories, P/S ratio > 1) lower serum total cholesterol levels by 10% or about 20 mg/dl serum (8). In normal humans the intake of large amounts of dietary cholesterol does not produce direct effects on plasma cholesterol because of regulation mediated by lipoprotein receptors (9). Carbohydrate-induced hypertriglyceridemia is well recognized, especially in subjects whose triglyceride levels already are elevated (10,11). Complex carbohydrates have a more transitory effect than simple ones on serum triglycerides (12). The effects of dietary treatment on serum HDL-cholesterol concentrations are less clear, and more information is needed (13). In short term feeding studies, high carbohydrate, low fat diets reduced serum HDL-cholesterol levels (14). Reports on the influence of changes in dietary polyunsaturated fat and cholesterol vary (13). The object of the experiments reported herein was to compare the effect of conventional foods simulating the usual American diet and of seven commercial defined formula diets (DFDs) of varying fat and carbohydrate content on lipid levels of healthy young adults.

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METHODS

General Design

The effect of DFDs on serum lipid parameters was investigated in three experiments, consisting of four or five dietary periods each seven days in length. Conventional foods (Table 1) were consumed during the initial period of all experiments and during the final period of Experiment 2. The DFDs, three per experiment (Table 1), were consumed in randomized order. Body weights were monitored daily throughout the study. Calorie intakes were adjusted to maintain subjects' initial body weight by altering intakes of cornstarch pudding (conventional food periods) or liquid formula. All diets were nutritionally complete. Subjects were allowed caffeine-free, sugar-free beverages and gum ad libitum.

Fasting blood samples were drawn by venipuncture on the first day of each experiment and on the morning following the comple-

TABLE 1

Composition of Experimental Diets

Diet	Percent of kilocalories			Cholesterol (mg)	P/S ratio
	Fat	Carbohydrate	Protein		
Conventional foods (CF)[a]	42	44	14	305	0.1
Moderate fat DFD[b] (MF1)	32 (corn oil)	53 (corn solids)	15 (casein, soy)	0	4.1
Moderate fat DFD (MF2)	32 (MCT,[c] corn and soy oil)	55 (Corn solids)	14 (casein, soy)	0	.67
Low fat DFD (LF1)	9 (safflower oil, MCT)	74 (glucose, oligo- and polysaccharides, sucrose)	17 (soy, whey and meat hydrolysate; EAA)[d,e]	0	.86
Low fat DFD (LF2)	9 (safflower oil, MCT)	74 (glucose, oligo- and polysaccharides, sucrose)	17 (soy, whey and meat hydrolysate; EAA)[d,e]	0	.86
Low fat DFD (LF3)	9 (safflower oil, MCT)	74 (glucose, oligo- and polysaccharides, sucrose)	17 (egg albumin and soy hydrolysate)	0	.86
Very low fat DFD (VLF1)	3 (safflower oil)	83 (maltodextrin, modified cornstarch)	14 (casein hydrolysate, AA)	0	5.5
Very low fat DFD (VLF2)	1 (safflower oil)	82 (glucose oligo-saccharides)	18 (AA)	0	5.5

[a]Basal CF female diet (grams/day): orange juice, 100; banana, 75; bread, 80; cornflakes, 15; jelly, 15; skim milk, 600; butter, 52; apricots, 100; pineapple, 50; lettuce, 30; cheese, 45; ice cream, 100; broccoli, 100; ground beef, 100; potatoes, 100; carrots, 40; peaches, 40; cornstarch pudding, sufficient to maintain body weight. Male portions were 38% larger.

[b]DFD = defined formula diet.

[c]MCT = medium-chain triglycerides.

[d]EAA = essential amino acids.

[e]LF1 and LF2 differed with regard to commercial source of meat hydrolysate.

tion of each dietary period. In Experiments 1 and 3 an adjustment day followed each dietary period. On the morning of the adjustment day subjects consumed a test meal of the moderate fat formula containing corn oil (MF1). Blood was sampled at intervals after feeding. Serum lipid levels during absorption of the fat load were monitored to assess the effect of the previous week's dietary adaptation. The subjects consumed the next period's diet for the remainder of the adjustment day.

Specific Study Design

Experiment 1. Experimental diets were the two moderate fat formulas, and a low fat formula, LF1 (Table 1). An adjustment day between dietary periods was included during which subjects received test meals providing 22 to 40 g of corn oil, depending on their usual calorie intake. Blood was sampled one and two hr after the meal.

Experiment 2. Experimental diets were LF1 and the very low fat formulas, VLF1 and VLF2. There was no adjustment day. Subjects consumed conventional foods during a five-day terminal period.

Experiment 3. Experimental diets were low fat formulas LF1, LF2 and LF3. On the last morning of each experimental dietary period (Day 7) all subjects consumed 600 ml (6 g fat) of their usual low fat formula for the period. The study included an adjustment day (Day 8) on which all subjects received a test meal of MF1 plus extra corn oil to provide a total of 50 g corn oil. On both Days 7 and 8, serum triglyceride levels were evaluated before and two hr after the test meal.

Subjects

Twelve healthy college students (6 males and 6 females) 19 to 33 years of age and within 10% of their desirable weight for height (Metropolitan Life Insurance Tables) participated in each experiment. Drugs other than mild analgesics were not taken. Subjects pursued normal activities during each experiment except that they ate carefully weighed, portion controlled meals in a metabolic unit. Prior to the study they signed consent forms approved by The Biomedical Sciences Human Subjects Review Committee of The Ohio State University.

Analyses

Serum total cholesterol was analyzed by a sequential enzyme procedure (Data Medical Associates, Arlington, Texas). Serum HDL-cholesterol levels were determined similarly after removal of other lipoprotein fractions by precipitation with phosphotungstate. The method was modified to measure free cholesterol by eliminating the cholesterol esterase step. Esterified cholesterol was estimated by difference. Serum triglycerides were measured by a colorimetric procedure (Sigma Chemical Company, St. Louis, Missouri, procedure no. 451). The ability of all blood sera samples obtained in Experiment 3 to activate lipoprotein lipase (LPL) was estimated by the method of Super et al. (15). LPL was prepared from fresh raw milk as a lyophilized powder after extensive dialysis at 4 C against deionized water. Tritium-labeled (2-^{3}H) glycerol triolein was the substrate in the LPL assay. Substrate and serum (2, 5 and 50 μl) were preincubated at 37 C for 30 min. Reconstituted skim milk was then added to the assay mixture and the reaction allowed to proceed for two hr. One milliliter of trichloroacetic acid (TCA) was added to stop enzyme activity and to precipitate unreacted substrate. The TCA supernatant containing labeled glycerol was then counted in a liquid scintillation counter.

Statistical Analyses

Summarization of main effects was by the Statistical Analytical System (SAS) or by Harvey's Mixed Model Least Squares and Maximum Likelihood Computer Program (16). Least squares means for treatment (diet) and period (week of the study) were adjusted, as appropriate, for several variables including gender, diet, gender × diet, and period (week of the study or diet rotation). When the experiments were balanced, least squares means equalled arithmetic means. Separation of means was by the Least Significant Difference (LSD) test after significant F was determined. Means were considered significantly different if $p < 0.05$.

RESULTS

Experiment 1 (Moderate and Low Fat Formulas)

Serum lipid levels of subjects are shown in Table 2. When an error term reflecting individual variations in serum lipid levels was used to test for gender differences in total and HDL-cholesterol and their ratio, no difference was significant. However, HDL-cholesterol levels were consistently higher in all female diet groups. Subjects had significantly lower serum

TABLE 2

Effect of Moderate Fat and Low Fat Defined Formula Diets on Serum Lipids (Experiment 1)[a]

Variable	Observations	Total cholesterol (mg/dl)	HDL-cholesterol (mg/dl)	Total:HDL cholesterol
Gender				
Males	24	180 ± 8	49.2 ± 2.0	3.78 ± 0.22
Females	24	187 ± 6	60.8 ± 3.1	3.33 ± 0.25
p		0.6346	0.1270	0.4878
Diet				
CF	12	207 ± 6[b]	62.1 ± 3.6[b]	3.46 ± 0.25[b]
MF1	12	158 ± 8[c]	55.7 ± 3.9[c]	3.00 ± 0.27[c]
MF2	12	183 + 7[d]	55.0 ± 4.0[c]	3.54 ± 0.31[bd]
LF1	12	185 ± 12[d]	47.0 ± 3.8[d]	4.22 ± 0.44[d]
p		0.0001	0.0001	0.0001

[a]Values are means ± standard errors.

[b-d]Means with common superscripts do not differ significantly ($p < 0.05$).

cholesterol levels when they consumed the moderate and low fat formulas rather than conventional foods. The moderate fat formula, MF1, with corn oil as the fat source resulted in significantly lower serum cholesterol levels than MF2 and LF1, both of which contained medium chain triglycerides (MCT) as well as polyunsaturated fat. Experimental period as well as diet had a significant ($p = .0025$) effect on serum total cholesterol levels of the subjects. Subjects who consumed MF1 during the first period of randomized formula feeding (immediately after CF) had significantly lower levels of serum total cholesterol than subjects consuming MF1 during the final week of formula feeding. A similar response was noted for LF1 but not for MF2. However, when mean serum cholesterol was adjusted statistically for the period effect, the MF1 diet still produced significantly lower total serum cholesterol levels than LF1 and MF2. HDL-cholesterol levels also were significantly lower when subjects consumed the moderate and low fat formulas rather than conventional foods. The low fat LF1 formula produced the greatest reduction (25%) in HDL-cholesterol levels. However, the ratio of serum total to HDL-cholesterol was significantly more favorable (lower) when MF1, the moderate fat DFD with 32% corn oil, was ingested. The least favorable (highest) ratio was seen when subjects consumed LF1, the low fat DFD. There was no significant period effect on HDL-cholesterol levels.

Consumption of LF1, in contrast to conventional food, resulted in significantly higher fasting serum triglyceride levels (Fig. 1). Females tended to have lower triglyceride levels than males. There was no significant period effect. Male subjects ingesting LF1 for one week appeared less able to clear the test fat load from their sera as evidenced by the higher triglyceride levels observed two hr after the fat load was administered (Fig. 1).

Experiment 2 (Low and Very Low Fat Formulas)

The effects of the low (9% and very low (1-3%) fat formulas and of conventional food on serum lipids were compared in Experiment 2. As in Experiment 1, when an error term reflecting individual differences in serum lipid levels was used in the statistical model to test for significant gender differences, there were none, although the higher average HDL-cholesterol levels observed in females consuming all diets (40.6 vs 32.4 mg/dl) approached significance ($p = 0.0879$).

Treatment, that is consumption of the low and very low fat formulas, resulted in significant changes in all serum lipid parameters except serum free cholesterol (Table 3). Serum total and HDL-cholesterol levels were reduced. Triglycerides and the ratio of total to HDL-cholesterol were raised. The 1% fat diet, VLF2, resulted in the lowest total and HDL-cholesterol levels.

Total and HDL-cholesterol levels were reduced more and triglyceride levels elevated most during the first week (period 2) of randomized formula feeding. Period differences among lipids during randomized formula feed-

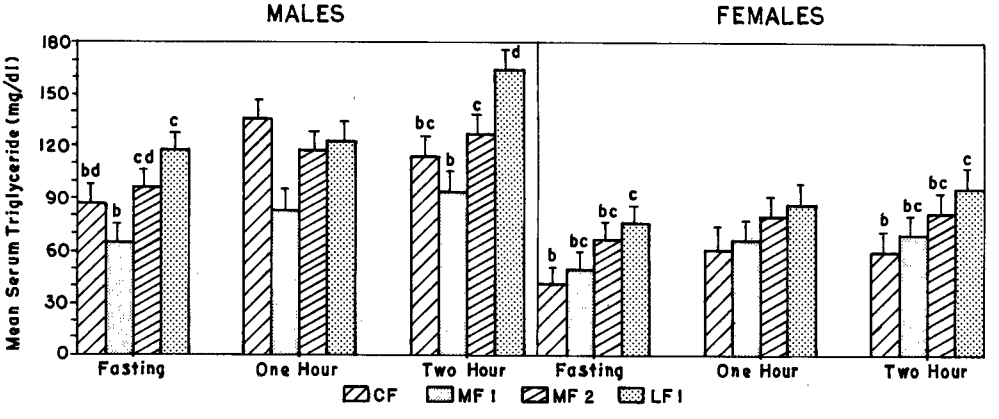

FIG. 1. Effect of adaptation to moderate fat and low fat defined formula diets on subjects' serum triglyceride levels before and after a test meal of constant composition (Experiment 1). Values are least squares means ±standard error of least squares means adjusted within gender group for constant test fat intake of 33 g for males and 22 g for females. Means within a gender group and time period having common superscripts or no superscripts do not differ significantly ($p < 0.05$) according to results of ANOVA.

TABLE 3

Influence of Low and Very Low Fat Defined Formula Diets on Serum Lipids (Experiment 2)[a]

Variable	Observations	Cholesterol (mg/dl) Total	Free	Esterified	HDL-cholesterol (mg/dl)	Total: HDL	Triglycerides (mg/dl)
Diet							
CF (period 1)	12	217 ± 8[b]	34 ± 2[bc]	183 ± 7[b]	46.3 ± 2.9[b]	4.91 ± 0.40[b]	75 ± 7[b]
LF1	12	184 ± 7[c]	36 ± 2[b]	148 ± 5[c]	33.0 ± 2.2[c]	5.85 ± 0.44[c]	166 ± 25[c]
VLF1	12	176 ± 7[c]	34 ± 1[bc]	142 ± 6[c]	31.1 ± 2.3[c]	5.98 ± 0.50[c]	168 ± 25[c]
VLF2	12	155 ± 9[d]	32 ± 2[c]	124 ± 6[d]	28.0 ± 2.5[d]	5.85 ± 0.49[c]	159 ± 22[c]
CF (period 5)	12	217 ± 8[b]	41 ± 2[d]	176 ± 7[b]	44.0 ± 2.4[b]	5.06 ± 0.30[b]	84 ± 8[b]
p		.0001	.0001	.0001	.0001	.0001	.0001

[a]Values are means ± standard errors.

[b-d]Means with common superscripts do not differ significantly ($p < .05$).

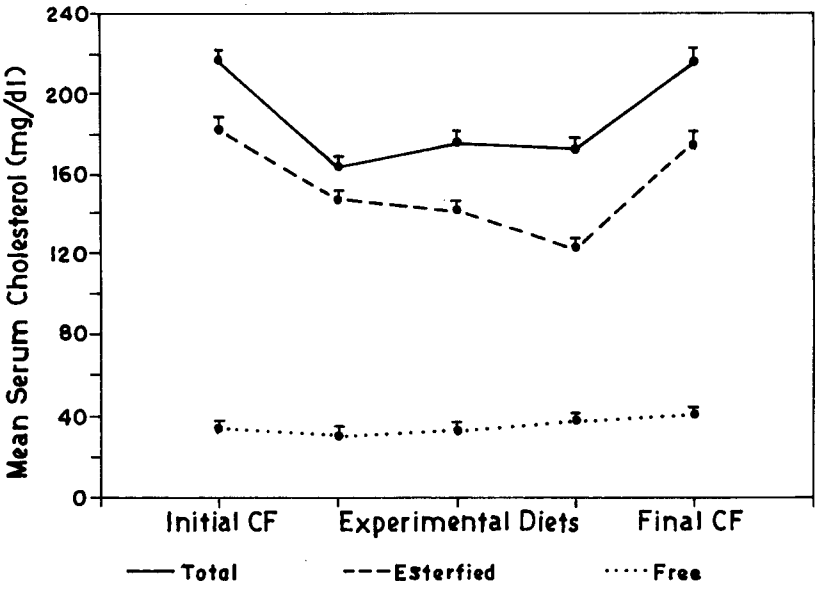

FIG. 2. Effect of period on serum total, free and esterified cholesterol levels of subjects consuming very low fat DFD during periods 2-4 and conventional foods during periods 1 and 5 (Experiment 2). Values are means ±SE.

ing (second through fourth weeks of the study) were significant for total, free and esterified cholesterol but not for HDL-cholesterol, the ratio of HDL to total cholesterol, or triglycerides. Free cholesterol tended to rise steadily from period 2 through period 5 (when conventional foods were resumed) (Fig. 2). Since serum esterified cholesterol levels were consistently reduced in subjects consuming the formulas, the ratio of esterified to free cholesterol declined during periods 2-4 and did not reach preformula values by the end of period 5. It is notable that with the exception of serum free cholesterol, all serum lipid parameters reverted back toward period 1 (conventional food) levels within five days of resumption of conventional foods (Fig. 2).

Correlations Between Serum Lipids (Experiments 1 and 2)

The simple Pearson correlations shown in Table 4 demonstrate that there was a consistent, moderate to strongly negative association between serum HDL-cholesterol and triglyceride levels and between the ratio of total to HDL-cholesterol and HDL-cholesterol levels in both Experiments 1 and 2. The correlations between total cholesterol and HDL-cholesterol and between total cholesterol and the total:HDL

TABLE 4

Correlations Between Selected Serum Lipid Parameters (Experiments 1 and 2)

Correlation variables	Experiment 1			Experiment 2		
	All	Males	Females	All	Males	Females
n	48	24	24	60	30	30
HDL-cholesterol						
Total cholesterol	−.04	.13	−.30	.60	.49	.65
Triglycerides (fasting)	−.54	−.35	−.60	−.68	−.77	−.56
Total:HDL cholesterol						
Total cholesterol	.64	.69	.70	.01	.12	.05
HDL-cholesterol	−.75	−.61	−.84	−.72	−.75	−.70
Triglycerides (fasting)	.68	.61	.77	.79	.83	.63

TABLE 5

Effect of Three Low Fat Defined Formula Diets on HDL-Cholesterol (Experiment 3)

Diet	Observations	HDL-cholesterol (mg/dl)		
		Total	Free	Esterified
CF	10	52.6 ± 1.0[b]	10.0 ± 0.2[b]	42.6 ± 1.0[b]
LF1	12	34.8 ± 0.9[c]	6.8 ± 0.2[c]	27.8 ± 0.9[c]
LF2	12	38.4 ± 0.9[d]	7.7 ± 0.2[c]	30.4 ± 0.9[cd]
LF3	12	40.0 ± 0.9[d]	7.3 ± 0.2[c]	32.3 ± 0.9[d]
P		.0000	.0000	.0000

[a]Least-squares means ± standard error of least-squares means adjusted for unequal subclass frequencies with respect to gender, individual males, individual females and gender X diet.

[b-d]Means with common superscripts do not differ significantly ($p < .05$).

cholesterol ratio were inconsistent between experiments, possibly due to inconsistent individual subject responses to the various experimental diets consumed. Serum triglycerides and total:HDL-cholesterol ratios were always moderately to strongly correlated. Male and female subjects did not differ appreciably.

Experiment 3 (Low Fat Formulas)

Interpretation of the results of this experiment was complicated by the fact that two male subjects dropped out at the end of the first (conventional food) period. They were replaced by two males who were not standardized on the conventional food diet. Since serum lipids of subjects on our other studies appeared to adjust rapidly to a new diet and the response of serum lipids did not appear to be particularly dependent on previous diet, the results of this experiment probably were not prejudiced by the replacement procedure.

Compared to conventional foods the three low (9%) fat diets produced significantly ($p = .0000$) lower levels of serum total and serum esterified cholesterol and significantly ($p = .0000$) higher levels of serum triglycerides. Serum free cholesterol, unlike serum esterified cholesterol, did not decline when the low fat DFDs were consumed so the ratio between the two fractions declined. These results were comparable to results reported for Experiment 2, so data are not shown. Both free and esterified HDL-cholesterol declined (Table 5) when subjects consumed the low fat formulas, so the ratio between the two was not appreciably altered.

Data from the Experiment 3 also were analyzed by period. As in Experiment 2, the maximal response of serum triglycerides and of the serum total and HDL-cholesterol fractions to diet occurred during the first low fat feeding period (period 2). Regardless of the particular low fat formula consumed, the cholesterol and HDL-cholesterol lowering effect and the triglyceride elevating effect of the low fat DFDs persisted for the remainder of the three weeks of randomized formula feeding. As in Experiment

TABLE 6

Effect of Independent Variables on Serum Lipids of Subjects Consuming Three Low Fat Defined Formula Diets (Experiment 3)[a]

Variable	Observations	Cholesterol (mg/dl)			HDL-cholesterol (mg/dl)			Triglycerides (mg/dl)
		Total	Free	Esterified	Total	Free	Esterified	
Day × State								
7 (fasting)	36	160 ± 2	42 ± 1	118 ± 2	37.2 ± 0.5	7.1 ± 0.1	30.3 ± 0.4	157 ± 5
7 (fed LF)	36	163 ± 2	44 ± 1	119 ± 2	37.5 ± 0.5	7.1 ± 0.1	30.4 ± 0.4	171 ± 5
8 (fasting)	36	166 ± 2	43 ± 1	123 ± 2	37.6 ± 0.5	7.5 ± 0.1	30.1 ± 0.4	156 ± 5
8 (fed test meal)	36	171 ± 2	47 ± 1	124 ± 2	37.4 ± 0.5	7.0 ± 0.1	30.4 ± 0.4	211 ± 5
p (day)		.0023	.0935	.0158	.7384	.4012	.8209	.0003
p (state)		.0652	.0064	.6049	.9274	.1142	.7220	.0000
p (gender × state)		.6725	.4144	.6703	.5240	.7793	.7710	.0178

[a]Least-squares means ± standard error of least-squares means adjusted, if necessary, for unequal subclass frequencies with respect to gender, period, diet, day, state, individual males, individual females, gender × diet, gender × day, gender × state, diet × day, diet × state and day × state.

2, in contrast to the other cholesterol fractions, serum free cholesterol levels tended to rise steadily and consistently during the experimental periods (2–4).

In Experiment 3, the effect of prior diet on the response of subjects' serum lipid levels to a test diet was assessed. On Day 7 subjects consumed a low fat test meal providing 6 g of fat in the form of their usual (for the period) low fat formula. On Day 8 subjects consumed a test meal providing 50 g of corn oil. Serum lipids were examined two hr after each meal. As expected, serum triglyceride levels were significantly affected by these procedures. The mean change in serum triglycerides between fasting and two hr was four-fold greater (+55 vs +14 mg/dl) after the 50-g fat load than after the 6-g fat load (Table 6). Feeding did not significantly affect any of the serum cholesterol parameters except for free cholesterol which was elevated slightly after both the 6-g and the 50-g fat loads even though these meals did not provide cholesterol. When fasting serum lipid values from Days 7 and 8 (Table 6) are compared, it can be seen that there was significant day to day variation in serum total and esterified cholesterol levels.

The influence of the dietary manipulations on serum lipids was similar for male and female subjects with one exception. As in Experiment 1, the increase in serum triglycerides between 0 and two hr was greater for males than females, resulting in a significant gender × state (fed vs fasting) interaction. Unlike Experiment 1, in which males received more fat in the test meal than females, males and females received the same fat load in Experiment 3.

Diet (conventional food vs low fat DFDs) did not significantly alter the ability of subjects' sera to activate LPL, so these data are not shown.

DISCUSSION

In these experiments serum lipid levels were studied in normal, free living subjects fed conventional foods simulating a typical American dietary pattern followed by three of seven nutritionally complete, moderate to low fat formulas. The low fat DFDs are often recommended for use by patients with maldigestive and malabsorptive states or by patients about to undergo diagnostic procedures or bowel surgery. Although it is unlikely that humans would normally consume DFDs for extended periods of time, the formulas fed in these experiments have about the same proportion of carbohydrate, fat and protein as CHD preventive diets advocated by some groups and individuals (17,18).

MF1, the moderate fat diet furnishing 32% of calories as corn oil, had the most favorable effect on serum lipids, producing the lowest serum cholesterol levels and lowest ratio of total to HDL-cholesterol. In contrast to conventional foods, MF2, the moderate fat diet with MCT, corn and soy oils, lowered total cholesterol by about 12% but did not lower the ratio of total to HDL-cholesterol. In a subsequent but similar (unpublished) experiment conducted in our facilities, MF1 lowered the ratio of total to HDL-cholesterol by 17% while MF2 produced only a 9% reduction when contrasted with the conventional food diet. High nitrogen versions of MF1 and MF2 produced 20% and

3% reductions, respectively. The difference between the effects produced by the two types of formula may be related to the higher P/S ratio of MF1 (4.1 vs .67) or to the MCT content of MF2. In contrast to long chain triglycerides, MCT are not transported in the chylomicron and are largely metabolized in the liver (19).

The diets producing the most marked effects were the low and very low fat DFDs which, in contrast to conventional foods, tended to reduce HDL-cholesterol levels by as much as 40%, to raise the total to HDL-cholesterol ratio by about 20%, and to raise serum triglycerides by as much as 100%. The effects of the low and very low fat DFDs were most pronounced during the first week of ingestion, but they showed evidence of persisting.

The HDL-cholesterol lowering effect of low fat, high carbohydrate diets has been noted by other investigators (14,19,21), some of whom have found that the reduction occurs in the HDL_2 fraction. The HDL-cholesterol lowering effect of the low fat diet fed by Brussard et al. (21) persisted for up to three mo.

HDL metabolism is related, at least in part, to triglyceride transport. According to Tall and Small (22) and Nikkila (23), serum HDL levels are a function of the splanchnic secretion rate of nascent HDL and non-HDL apoprotein A-1, the breakdown rate of the A apolipoproteins, and the rate of catabolism of high triglyceride lipoproteins. Low HDL-cholesterol levels are associated with a low fractional removal rate of triglycerides from the serum (24) and with elevated serum triglycerides (23). The results of these experiments provide more evidence for the negative association between serum triglycerides and HDL-cholesterol. These studies also provide evidence, although the data are not always statistically significant, that males and females differ in regard to the extent to which low fat diets influence triglyceride metabolism.

Various investigators have examined the effect of low fat, high carbohydrate diets on apolipoproteins. Schonfeld et al. (19) reported that the proportion of apo C-2 increased relative to apo C-3 in subjects fed low fat diets. Brussard et al. (21) reported that the apo C-2/C-3 ratio in VLDL from subjects consuming low (20% of calories) fat diets did not change. The findings reported here agree with Brussard et al. in that lipoprotein lipase activation was not influenced by the low fat diet as it might have been if the apo C-2/C-3 ratio changed.

There is evidence that apo A-1 synthesis is influenced by fat absorption (25), probably because apo A-1 synthesis is associated with chylomicron formation in the small intestine. Schonfeld et al. (19) showed that when subjects consumed a very low fat diet, lower (16%) plasma apo A-1 levels resulted. The fractional catabolism of A apoproteins is increased when subjects consume low fat, high carbohydrate diets (14).

It is quite possible that the effects observed in this study were due to the low fat content of the low fat formulas rather than to their high carbohydrate. Theoretically, the greater insulin response elicited by high carbohydrate diets should result in greater LPL activity and hence elevated fat clearance. Since fat clearance apparently was reduced when the low fat diets were consumed, the reduced fat absorption may have resulted in reduced chylomicron formation and hence lower apo A-1 levels. The reduction in cholesteryl ester relative to free cholesterol and the lack of effect of low fat LF1 on LPL activation were consistent with this concept.

In summary, the data reported herein show that a cholesterol-free, moderate fat diet with polyunsaturated fat produced favorable changes in serum lipoprotein parameters associated with CHD. Low and very low fat diets tended to reduce serum HDL-cholesterol levels more than total cholesterol levels. These changes are not of great importance to patient populations who must consume such diets as part of their care. However, the American Heart Association currently is suggesting that people with blood lipid abnormalities reduce their fat intake to 20% of calories in three phase therapy (26). Brussard et al. (21) observed effects on serum lipids similar to the ones described herein when a diet providing 20% of calories as fat was fed. The effects of conventional food diets with low fat content should be investigated in long-term controlled metabolic experiments before such diets are recommended to the general population for CHD or cancer prevention.

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Suppression of Cholesterogenesis by Plant Constituents: Review of Wisconsin Contributions to NC-167

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ABSTRACT

In animals, non-sterol metabolites of the mevalonate pathway act independently from receptor-mediated cholesterol uptake in the multivalent feedback regulation of mevalonate biosynthesis. Studies leading to the isolation and characterization of plant-borne suppressors of mevalonate biosynthesis are reviewed. We propose that one cardio-protective component of the vegetarian diet consists of a variety of non-sterol, post-mevalonate metabolites. These products of plant branches of the mevalonate pathway, discarded as animals evolved, continue to influence animal sterol metabolism. It is through this action, we propose, that the cholesterol-suppressive action of plant materials is expressed.
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Epidemiological data covering several populations indicate that vegetarians and other individuals who consume diets principally of plant origin are at lower risk for cardiovascular disease (1-4). A shift from traditional Western dietary patterns toward the dietary pattern of vegetarians is recommended for the American population (5). In experimental animals, a low-cholesterol or high-fiber diet that lowers plasma cholesterol tends to increase endogenous cholesterol synthesis. Cholesterol synthesis can be decreased by increasing cholesterol intake (6), an approach which maintains (7,8) or elevates plasma cholesterol levels (9,10). A new approach to the study of dietary influence on cholesterol metabolism is suggested by O'Brien and Reiser (11,12). They note that factors which elevate plasma cholesterol are more potent when added to a purified diet. This observation implies that crude diets contain factors which either dampen the effects of hypercholesterolemic factors or which act apart from cholesterol in suppressing cholesterol biosynthesis. Brown and Goldstein (13) propose that the full suppression of cholesterol biosynthesis requires two regulators, cholesterol derived from LDL and a non-sterol product(s) derived from mevalonate, both of which modulate 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity. Plants, presenting a complex series of pathways stemming from mevalonate, provide a broad variety of non-sterol metabolites. Our studies show that some of these metabolites suppress HMG-CoA reductase activity and, concomitantly, lower plasma LDL cholesterol levels of experimental animals.

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The studies reviewed in this report have roots in experiments in which the feed value of cereal grains was improved by the addition of a commercial β-glucanase to poultry rations (14-16). Diets modified with the isonitrogenous substitution of cereal grains for corn and soybean meal failed to support weight gains equal to those obtained by feeding a standard corn-soy diet. As has been observed in studies of other species (17,18), day-old birds fed the fiber-rich cereal grains for three weeks had lower plasma cholesterol levels, an effect which might be attributed to an increased excretion of cholesterol metabolites. Based on reports of its response to cholestyramine (19,20), we anticipated that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity would be considerably elevated in livers of birds which had been fed barley, oats or rye. To the contrary, we (15) found substantially lower HMG-CoA reductase activities in these groups (Table 1). These observations suggest that the hypocholesterolemic action of the cereal grains cannot be attributed solely to a fiber-enhanced excretion of cholesterol metabolites. Barley exerted a second effect on lipid metabolism. Fatty acid synthetase (FAS) activity in livers of fasted-refed birds fed the barley-based diet was 5-fold that present in birds which had been fed the corn-based diet (Table 1). This conclusion gained support in a second study in which pigs were fed diets containing either corn or barley. Barley proved to be equal to corn in supporting weight gain (21). Serum cholesterol levels of pigs fed the corn-based diet did not change over the 21-day trial; in those pigs fed the barley-based diet the serum cholesterol level was decreased by 18%. A similar difference was recorded in the cholesterol concentrations of the

TABLE 1

Influence of Cereal Grains on Avian[a] Lipid Metabolism (15)

Diet	Body weight	Plasma	Hepatic enzymes	
	g	Cholesterol mg/100 ml	HMG-CoA reductase[c]	FAS[d]
Corn	272 ± 7	139 ± 7	2.9 ± 0.6	5.0 ± 0.5
Wheat[b]	221 ± 9	106 ± 4	2.2 ± 0.4	11.2 ± 1.7
Barley[b]	188 ± 6	76 ± 4	0.6 ± 0.1	26.2 ± 2.4
Oats[b]	195 ± 7	95 ± 4	1.2 ± 0.3	7.7 ± 1.4
Rye[b]	166 ± 9	105 ± 5	1.1 ± 0.2	6.7 ± 0.4

[a]1-day old mixed sex, White Leghorn chicks, fed 18 days, fasted 2 days, refed 3 days.

[b]Isonitrogenous cereal grain substitution for corn/soy components of diet.

[c]nmol mevalonate/g liver/min.

[d]μmol NADPH oxidized/g liver min.

TABLE 2

Influence of Commercial Milling Fractions of Barley on Avian[a] Lipid Metabolism (22)

Isonitrogenous diet	Body weight	Plasma	Hepatic enzymes		
	g	Cholesterol mg/100 ml	HMG-CoA reductase[b]	Cholesterol 7α-hydroxylase[c]	FAS[d]
Corn	260 ± 5	165 ± 18	5.5 ± 0.4	28.0 ± 3.0	3.4 ± 0.1
Barley	249 ± 6	128 ± 12	2.3 ± 0.9	20.9 ± 0.9	12.1 ± 0.4
Barley Pearlings	112 ± 10	121 ± 10	3.4 ± 0.8	16.7 ± 3.0	12.3 ± 0.7
HPBF	205 ± 14	115 ± 12	2.1 ± 0.2	17.9 ± 4.0	15.7 ± 0.6
Pearled Barley	210 ± 13	130 ± 11	3.5 ± 0.3	21.7 ± 3.0	12.3 ± 0.3

[a]14-day old White Leghorn pullets, fed 9 days, fasted 2 days, refed 3 days.

[b]nmol mevalonate/g liver/min.

[c]pmol 7α-hydroxycholesterol/g liver/min.

[d]μmol NADPH oxidized/g liver/min.

semimembranous muscles. The pig differs from the chicken not only in the anatomy of the digestive tract but also in the distribution of the lipogenic enzymes. Under fed conditions, HMG-CoA reductase activities were lower and FAS activities higher in liver, adipose tissue, intestine, lung and muscle of pigs which had received the barley-based diet (21).

A progression of experiments led to the isolation of three constituents of barley, each of which suppressed mevalonate biosynthesis and, concomitantly, lowered serum cholesterol levels. Initially, the three commercial milling fractions of barley and barley were incorporated into diets calculated to be isonitrogenous with the corn-soy diet (22). These diets were fed ad lib for a two-week period to 14-day old chicks. Responses to barley shown in Table 2 are of the same magnitude as those recorded in Table 1, except in regard to body weights. This difference may be due to maturation of the digestive tract prior to the introduction of the barley-based diet. Cholesterol 7 α-hydroxylase activity also was lower (Table 2). This observation supports our view that the cholesterol-suppressive action of barley is not solely a function of increased metabolite excretion.

Diets containing the commercial milling fractions failed to support weight gains equal to those produced by feeding barley (Table 2). Yet, FAS activity was elevated by these regimens. The effectors of serum cholesterol level, HMG-CoA reductase and cholesterol 7-α-hydroxylase failed to segregate into a single milling fraction. HPBF (high protein barley flour), the fraction consisting of the aleurone, subaleurone and germ, is a light, fine-powdered material. When fed as a wet-mash, HPBF proved to be equal to corn in supporting weight gain (22). Also, when fed in partial replacement of corn, HPBF

TABLE 3

Influence of HPBF on Avian[a] Lipid Metabolism (22)

Diet	Body weight	Hepatic enzymes	
	g	HMG-CoA reductase[c]	FAS[d]
Corn	491 ± 15	3.8 ± 0.3	1.5 ± 0.3
Barley[b]	463 ± 12	1.0 ± 0.2	3.0 ± 0.4
Corn, 5% HPBF	508 ± 15	3.3 ± 0.3	2.2 ± 0.1
Corn, 10% HPBF	538 ± 20	2.1 ± 0.2	4.2 ± 0.2
Corn, 15% HPBF	573 ± 26	1.7 ± 0.2	4.6 ± 0.1
Corn, 20% HPBF	600 ± 23	1.4 ± 0.3	5.3 ± 0.2

[a]14-day old White Leghorn pullets, fed 27 days, fasted 2 days, refed 3 days.
[b]Isonitrogenous.
[c]nmol mevalonate/g liver/min.
[d]μmol NADPH oxidized/g liver/min.

TABLE 4

Influence of Serial-Solvent Fractions of HPBF (20% equivalent) on Avian[a] Lipid Metabolism (23)

Diet[b]	Body weight (g)		Serum	Hepatic enzymes		
	Initial	Final	Cholesterol mg/100 ml	HMG-CoA reductase[c]	Cholesterol 7 α-hydroxylase[d]	FAS[e]
Corn	680 ± 57	1010 ± 92	208 ± 8	690 ± 44	6.7 ± 0.2	62 ± 8
20% HPBF	643 ± 48	983 ± 63	178 ± 9	460 ± 42	3.7 ± 0.2	72 ± 7
0.7% PESF[g]	603 ± 42	1008 ± 49[f]	164 ± 7	440 ± 37	5.0 ± 0.4	37 ± 3
0.5% EASF[h]	676 ± 65	979 ± 61	204 ± 10	460 ± 38	5.5 ± 0.4	113 ± 16
0.8% MESF[i]	634 ± 58	924 ± 76	159 ± 12	350 ± 37	3.4 ± 0.5	121 ± 14
1.7% WASF[j]	608 ± 51	920 ± 94	202 ± 5	370 ± 32	4.1 ± 0.4	151 ± 12
16.3% Residue	653 ± 56	970 ± 51	201 ± 6	630 ± 43	4.6 ± 0.5	141 ± 11

[a]9-wk old White Leghorn pullets, fed 16 days, fasted 2 days, refed 3 days.
[b]Nearly isonitrogenous with corn-soy diet.
[c]pmol mevalonate/mg microsomal protein/min.
[d]pmol 7 α-hydroxycholesterol/mg microsomal protein/min.
[e]nmol NADPH oxidized/mg cytosolic protein/min.
[f]Mean weight gain, 405 g significantly greater than all other values.
[g]Petroleum ether soluble fraction.
[h]Ethyl acetate soluble fraction.
[i]Methanol soluble fraction.
[j]Water soluble fraction.

elicited dose-dependent (to 20% of diet) increases in weight gain and FAS activity and a decrease in HMG-CoA reductase activity (Table 3).

The isolation of the constituents responsible for these effects began with the serial extraction of HPBF with solvents of increasing polarity (23). These fractions and the residue were fed to 9-week old pullets at levels equivalent to 20% HPBF for three weeks. Diets containing the soluble fractions were essentially isonitrogenous with the corn-soy diet; diets containing 20% HPBF and the residue were not adjusted for nitrogen content. The HPBF constituent(s) which suppressed mevalonate biosynthesis and cholesterol oxidation did not segregate into a single solvent fraction (Table 4). The more polar extracts contained factors which enhanced FAS activity; a non-polar constituent of the petroleum ether extract strongly suppressed this activity. Petroleum ether and methanol extracts effectively lowered the serum cholesterol level. The responses to these extracts were confirmed in a study that yielded the additional observation that the reduction of serum cholesterol occurred primarily in the LDL fraction (Table 5). Hepatic FAS activity and serum triglyceride level were elevated by the more polar extract

TABLE 5

Influence of Serial Solvent Fractions of HPBF (20% equivalent) on Avian[a] Lipid Metabolism (23)

Diet	Body weight (g)	Serum				Hepatic enzymes		
		Cholesterol (mg/100 ml)			Triglyceride (mg/100 ml)	HMG-CoA[c] reductase	Cholesterol 7 α-hydroxylase[d]	FAS[e]
		Total	HDL	LDL				
Corn	1050 ± 30	157 ± 11	57 ± 7	78 ± 6	140 ± 5	690 ± 47	13.6 ± 0.6	82 ± 9
20% HPBF	1216 ± 20	141 ± 13	50 ± 6	62 ± 5	146 ± 7	500 ± 42	6.2 ± 0.3	126 ± 7
0.7% PESF[b]	1270 ± 25	121 ± 9	47 ± 6	53 ± 6	111 ± 4	470 ± 43	4.1 ± 0.2	31 ± 3
0.8% MESF[b]	1056 ± 30	117 ± 11	48 ± 7	34 ± 5	174 ± 6	346 ± 34	3.4 ± 0.2	151 ± 12

[a]21-day old cross-bred broiler pullets, fed 23 days, fasted 2 days, refed 3 days.

[b]Nearly isonitrogenous with corn-soy diet.

[c]pmol mevalonate/mg microsomal protein/min.

[d]pmol 7 α-hydroxycholesterol/mg microsomal protein/min.

[e]nmol NADPH oxidized/mg cytosolic protein/min.

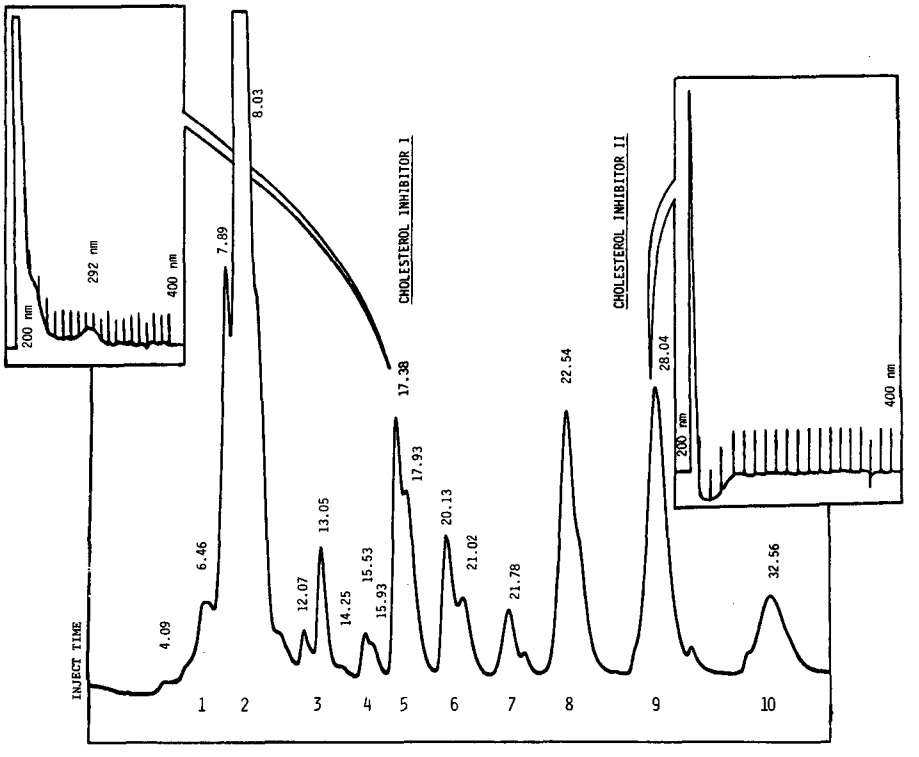

FIG. 1. HPLC elution profile of the petroleum ether solubles of HPBF. The components were eluted from a Beckman Ultrasphere C_{18} IP column (25 cm × 4.6 mm ID, 10μ) with methanol at a flow rate of 1 ml/min (700 psi). The detecting wavelength was 200 nm. Each peak was scanned over the range 200–400 nm.

and lowered by the less polar extract. In comparison with controls, chicks fed the 20% HPBF diet gained more weight and had higher FAS activity (Tables 3-5). These effects were not significant in the older birds (Table 4). This activity also was higher when HPBF was fed as the major energy source (Table 2). The recordings of a lower (Table 2) and of higher gains in weight (Tables 3-5) appear to dissociate an increased FAS activity from increased growth. This dissociation is confirmed (Tables 4,5) in that the petroleum ether soluble fraction contained a factor(s) which increased growth and suppressed FAS activity. This effect of the petroleum ether extract of HPBF has since been confirmed in rats and pigs. Feed efficiency, increase in body weight per unit feed consumed, also was increased. Although body compositions have not been determined, calculations based on back fat, length and loin eye measurements point to a higher lean/fat ratio for carcasses of swine which had been fed this extract.

Analysis of HPBF yields 18.1% protein, 15.5% pentosans, 8.1% acid detergent fiber and 4.7% ether extractables (22). Presumably, all components except the latter are retained in the residue (Table 4), which had no influence on the cholesterol levels of chicks fed a cholesterol free diet. Polar and apolar extracts of HPBF, on the other hand, elicited responses in LDL cholesterol concentration similar to those attributed to cereal fibers in studies of hypercholesterolemic men (24) and young normal men (25).

The HPLC elution profile of the petroleum ether extract of HPBF is shown in Figure 1. When tested in an avian hepatocyte system, two components, fractions 5 and 9 (Fig. 1), were found to suppress HMG-CoA reductase activity (Table 6). Fraction 5 increased FAS activity, whereas fraction 9 suppressed this activity. Cholesterol-suppressive metabolites have been isolated from HPLC fractions 5 and 9 (details printed elsewhere). The molecular ion (M^+ 424) and major peaks at m/e 205, 203 and 165 of the fraction 5 metabolite are characteristic of d-α-tocotrienol. HPLC fraction 9 yielded three major components. The mass spectrum of the metabolite which most effectively suppressed FAS and HMG-CoA reductase activity showed a fragmentation pattern characteristic of a triglyceride with a molecular ion peak at M^+ 876. The fatty acid pattern consists of 66% linoleic acid and 33% linolenic acid.

Other plant materials are reported to have cholesterol-suppressive actions which cannot be attributed to their fiber components. Garlic paste (3.8 g) prepared from 5 g garlic bulbs and its equivalent serial solvent fractions were fed to chicks at the levels indicated in Table 7. Garlic paste and each of the serial solvent fractions contained constituents which suppressed HMG-CoA reductase and cholesterol 7 α-hydroxylase activities; serum total and LDL cholesterol levels were lowered. Each of the solvent fractions contained constituents which suppressed FAS and other lipogenic activities (26). The residue had no influence on these parameters (Table 7). The polar solvent fraction of garlic paste, tested in vivo, effected dose-dependent suppressions of HMG-CoA reductase and cholesterol 7 α-hydroxylase activities and lowered LDL cholesterol (27). These extracts also suppress HMG-CoA reductase and FAS activities in avian hepatocytes. The sup-

TABLE 6

Influence of HPLC Fractions of PESF-HPBF on Avian Hepatocyte Lipid Metabolism (26)

HPLC fraction[a]	HMG-CoA reducatase: pmol mevalonate/mg protein/min	FAS: nmol NADPH oxidized/mg protein/min
Control	75.9(100)	63(100)
1	74.2	69
2	78.4	69
3	86.8	71
4	64.9	72
5	24.6(32)[b]	87(138)[b]
6	74.0	61
7	68.9	57
8	70.8	62
9	31.3(41)[b]	42(67)[b]
10	73.7	65

[a]See Fig. 1.
[b]% of control.

TABLE 7

Influence of Garlic on Avian[a] Lipid Metabolism (26)

Diet	Serum (mg/100 ml)				Hepatic enzymes		
	Cholesterol						
	Total	HDL	LDL	Triglyceride	HMG-CoA[b] reductase	Cholesterol 7 α-hydroxylase[c]	FAS[d]
Corn	168 ± 20	57 ± 4	86 ± 7	125 ± 12	909 ± 70	1.14 ± 0.08	175 ± 14
3.8% garlic paste	133 ± 10	56 ± 8	58 ± 5	98 ± 10	253 ± 40	0.72 ± 0.04	158 ± 12
0.014% PESF[e]	129 ± 8	56 ± 8	59 ± 4	113 ± 10	193 ± 30	0.61 ± 0.03	124 ± 11
0.341% MESF[f]	127 ± 4	57 ± 5	51 ± 4	93 ± 11	159 ± 28	0.56 ± 0.04	141 ± 11
0.79% WASF[g]	135 ± 11	55 ± 6	61 ± 5	96 ± 10	192 ± 28	0.56 ± 0.05	145 ± 8
0.121% residue	152 ± 14	54 ± 4	74 ± 7	122 ± 10	781 ±62	0.93 ± 0.06	172 ± 13

[a]56-day old White Leghorn pullets, fed 24 days, fasted 1 day, refed 3 days.
[b]pmol mevalonate/mg microsomal protein/min.
[c]pmol 7 αhydroxycholesterol/mg microsomal protein/min.
[d]nmol NADPH oxidized/mg cytosolic protein/min.
[e]Petroleum ether soluble fraction.
[f]Methanol soluble fraction.
[g]Water soluble fraction.

TABLE 8

Influence of Chinese Red Ginseng on Avian[a] Lipid Metabolism (29)

Diet	Serum			Hepatic enzymes		
	Cholesterol					
	Total	HDL	LDL	HMG-CoA reductase[b]	Cholesterol 7 α-hydroxylase[c]	FAS[d]
Corn	165 ± 10	56 ± 4	83 ± 7	595 ± 90	1.9 ± 0.04	145 ± 9
0.284% *ginseng*	134 ± 8	55 ± 4	53 ± 6	284 ± 65	1.1 ± 0.002	137 ± 7
0.032% PESF[e]	128 ± 9	56 ± 4	51 ± 7	188 ± 42	0.8 ± 0.02	115 ± 4
0.098% MESF[f]	148 ± 7	53 ± 4	70 ± 7	349 ± 60	1.5 ± 0.03	132 ± 5
0.084% WASF[g]	130 ± 7	56 ± 3	51 ± 6	218 ± 58	0.9 ± 0.01	122 ± 4
0.062% residue	159 ± 9	53 ± 4	73 ± 7	539 ± 75	1.8 ± 0.03	142 ± 9

[a]28-day old cross-bred broiler pullets, fed 23 days, fasted 2 days, refed 3 days.
[b]pmol mevalonate/mg microsomal protein/min.
[c]pmol 7 αhydroxycholesterol/mg microsomal protein/min.
[d]nmol NADPH oxidized/mg cytosolic protein/min.
[e]Petroleum ether soluble fraction.
[f]Methanol soluble fraction.
[g]Water soluble fraction.

pression is both time and concentration dependent (27). The effector in the methanol solubles has been isolated, and some of its structural characteristics have been determined. The molecular ion (M^+ 358) and other major peaks were at m/e 343, 284, 268, 252, 205 and 188, which are characteristic of a molecule consisting of one isoprenoid unit and a quinone ring.

A brief report of the effects of Wisconsin ginseng root on human cholesterol metabolism (28) prompted us to feed chicks diets containing 0.25% Wisconsin ginseng root or 0.248% Chinese red ginseng root or their equivalents in serial solvent fractions (29). The avian responses to Chinese red ginseng constituents (Table 8) are generally in accord with those of the petroleum ether solubles of HPBF and differ from the garlic solubles only in action of increasing body weight gain (29).

Correlations drawn from data recorded in the publications cited above suggest highly significant relationships between HMG-CoA reductase activity and cholesterol 7 α-hydroxylase activity ($r = 0.934$), total serum cholesterol level ($r = 0.880$) and serum LDL cholesterol

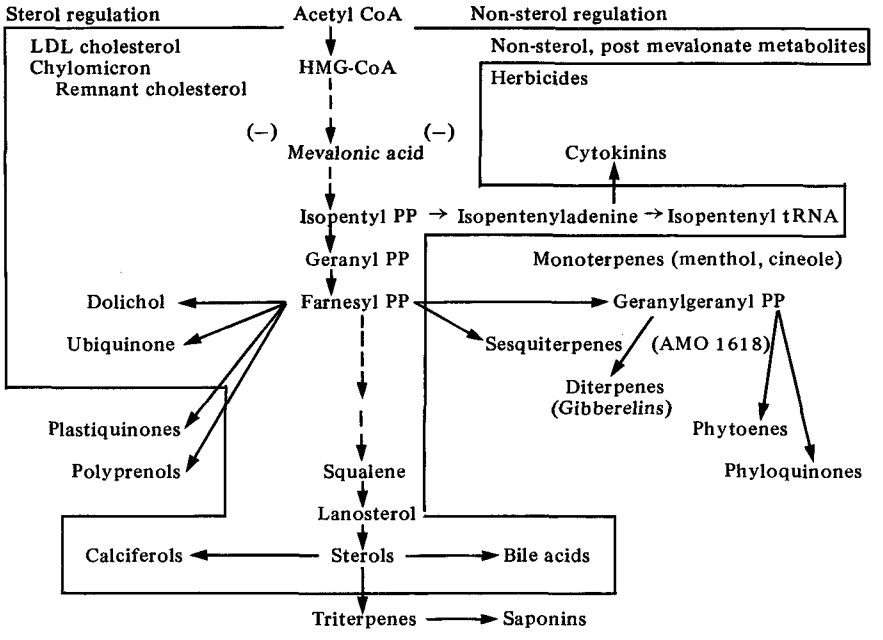

FIG. 2. Overview. As far as we can determine, all metabolites within the enclosed space (animal), except LDL and chylomicron remnant cholesterol and the bile acids, are present in plant tissues. We propose that sterol regulation is limited to animal systems and non-sterol regulation is shared by both plants and animals.

level ($r = 0.868$). On the other hand, the correlation of HMG-CoA reductase activity with HDL cholesterol level is only $r = 0.019$. These relationships point to the impact of HMG-CoA reductase activity on LDL cholesterol levels of avians which were fed a diet essentially free of cholesterol. Whether such relationships can be extended to a human population consuming a cholesterol-rich diet has yet to be determined.

In our studies we have shown that minor constituents of plant materials influence cholesterol metabolism. Two of the plant constituents contain fragments which are products of post-mevalonate pathways in plants. Other products of plant post-mevalonate pathways, menthol and cineole (Fig. 2), suppress HMG-CoA reductase activity (30). Clegg et al. (30) describe the concentration-dependent reduction of HMG-CoA reductase due to the decrease in enzyme mass following the in vivo administration of menthol to both fed and fasted rats. Since the inhibitory action could be demonstrated in fasted rats, the implication is that the rate of HMG-CoA reductase degradation is enhanced.

Edwards et al. (20) propose that post-mevalonate cellular products accumulating due to a block in cholesterol biosynthesis destabilize HMG-CoA reductase which results in an enhanced rate of its degradation. Sterol biosynthesis in animals shares in common part of the post-mevalonate pathway of plants which yield, among other secondary metabolites, the plant growth hormone, gibberellic acid (Fig. 2). AMO 1618, a plant growth retardant, inhibits the cyclization of geranylgeranyl pyrophosphate in the biosynthesis of the gibberellins (31). We fed AMO 1618 to chicks (2.5–15 ppm) and rats (20 ppm) for 21 days and 3 days, respectively (32). In both species, AMO 1618 caused decreases in HMG-CoA reductase and cholesterol 7 α-hydroxylase activities and in plasma total and LDL cholesterol levels (Table 9).

When multiple end-products are formed through a common sequence of reactions in a biosynthetic pathway, an end-product inhibition by an excess of one product might be expected to decrease not only its own production but also that of other products derived from a common precursor. It seems reasonable that the introduction of post-mevalonate products of plant metabolism into an animal diet will suppress mevalonate biosynthesis and hence, the synthesis of the major end-product, cholesterol, as the available mevalonate is directed toward higher affinity pathways.

We propose that, for the normal population, the protective component of the vegetarian diet consists, in part, of a variety of non-sterol, post-

TABLE 9

Influence of a Plant Growth Retardant, AMO 1618, on Lipid Metabolism (32)

	Chickens[a]						Rats[b]	
AMO 1618 (ppm)	0	2.5	5.0	7.5	10	15	0	20
Plasma cholesterol								
Total mg/100 ml	192	177	162	158	156	150	98	72
LDL mg/100 ml	90	73	64	56	44	41	59	31
HDL mg/100 ml	80	77	70	68	66	63	30	27
Hepatic enzymes								
HMG-CoA reductase[c]	430	370	343	320	262	237	370	280
Cholesterol 7 α-hydroxylase[d]	0.85	0.67	0.58	0.46	0.45	0.27	4.9	1.6
FAS[e]	45	93	101	122	133	150	149	591

[a]63-day old White Leghorn pullets, fed 16 days, fasted 1 day, refed 3 days.

[b]57-day old Sprague-Dawley males fed 3 days.

[c]pmol mevalonate/mg microsomal protein/min.

[d]pmol 7 α-hydroxycholesterol/mg microsomal protein/min.

[e]nmol NADPH oxidized/mg cytosolic protein/min.

mevalonate metabolites which act in the regulation of HMG-CoA reductase at the non-sterol site. These metabolites are, we suggest, distributed among the secondary products of plant metabolism whose roles in human nutrition, other than as the fat-soluble vitamins or their precursors, have generally escaped investigation.

ACKNOWLEDGMENT

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Cholesterol Metabolism in Relation to Aging and Dietary Fat in Rats and Humans[1]

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ABSTRACT

A review of research in the authors' laboratories regarding effects of dietary fat polyunsaturation upon longevity in rats and some aspects of the regulation of cholesterol metabolism with regard to age of rats and humans is presented. The longevity of the rat was found to be enhanced by consumption of dietary fat providing a polyunsaturated to saturated fatty acid (P/S) ratio of 0.3 to 1, corresponding to about 5–12% of energy (en%) as linoleate, compared with less or more polyunsaturated fat. Mechanisms of the effects of the fats upon cholesterol metabolism were studied. With advancing age, there seems to be a decline in the rate of catabolism of cholesterol, resulting in longer retention in the body of the rat. In the human, there seems to be a decline in regulation of uptake of cholesterol by leukocytes and, therefore, perhaps other tissues, resulting in increased synthesis of cholesterol by the peripheral tissues. Moderate rather than high dietary consumption of polyunsaturated fat seems to be favorable to metabolic processes contributing to longevity. *Lipids* 20:825–833, 1985.

INTRODUCTION

There has been a continuing quest for sufficient information to know exactly how much and what kind of fat in the diet are most conducive to good health and long life. This review will discuss studies conducted in our laboratories on aging (1–3), which have been designed to determine an optimum dietary intake of essential fatty acids. Studies that have been conducted to determine possible mechanisms of dietary fat effects upon cholesterol and bile acid metabolism are included (4–6). Methods for those studies have been published in the papers cited.

For further insight into mechanisms of regulation of cholesterol metabolism, we have utilized the leukocyte as a model for metabolism in peripheral tissues. The original research conducted to verify the applicability of the leukocyte model is reported herein. Application of the use of the leukocyte was made in a human study of aging (7), and those results are included in this review.

MATERIALS AND METHODS

Animals

Male rats of the Sprague Dawley strain, weighing from 200–250 g initially, were used in the rat leukocyte experiments (SASCO, NE or ARS, Madison, Wisconsin). Lighting of the rat rooms was set so the dark phase occurred between 6:00 and 18:00 hr, and the light phase occurred between 18:00 and 6:00 hr, or vice versa. This facilitated experimentation with the highest diurnal enzyme activity and the lowest at the same time. All rats were fed ad libitum and given deionized water.

Diets

The composition of the four purified treatment diets is listed in Table 1. All diets contained 40% of energy (en%) as fat; however, the

TABLE 1

Composition of Diets used in the Rat Leukocyte Study

	BT	BT + CHOL	SO	SO + CHOL
	(Weight percent)			
Lactalbumin	19.00	19.00	19.00	19.00
Cornstarch	25.30	22.30	25.30	22.30
Sucrose	25.00	25.00	25.00	25.00
Cellulose	2.50	2.50	2.50	2.50
Vitamin mix[a]	2.00	2.00	2.00	2.00
Mineral mix[a]	5.00	5.00	5.00	5.00
Soybean oil (SO)[b]	4.29	4.29	20.52	20.52
Beef tallow (BT)[c]	16.19	16.19	0.68	0.68
Cholesterol (CHOL)[d]	0	3.00	0	3.00
P/S ratio	0.34	0.34	3.81	3.81

[a]Ref. 2.
[b]Nobel Supreme Salad Oil, Denver, CO.
[c]Litwak Rendering Co., Denver, CO.
[d]ICN Pharmaceuticals, Inc., Cleveland, OH.

[1]Presented at the AOCS meeting in Chicago, May 1983.

type of fat used in each diet was varied to yield a polyunsaturated to saturated (P/S) ratio as follows: predominantly beef tallow (BT), P/S = 0.34, and predominantly soybean oil (SO), P/S = 3.81. Half of the diets were made hypercholesterogenic (CHOL) by substitution of 3% cornstarch with cholesterol by weight. Addition of cholesterol to the diets was accomplished by first melting the appropriate fat and then dissolving the cholesterol in the melted fat (8). After cooling, the fat-cholesterol mixture was added to the diet. Each diet was kept frozen until it was fed to the rats.

TABLE 2

Cholesterol (CHOL) and Triglyceride (TG) Content of Diluted Sera used for Incubation of Leukocytes

	Lipid-full		Lipid-poor	
Diet[a]	CHOL	TG	CHOL	TG
	(mg/dl)			
BT	55	59	22	22
BT + CHOL	72	83	28	34
SO	55	72	26	32
SO + CHOL	58	83	24	32

[a]BT, diet containing 40 en% beef tallow and 2 en% soybean oil; SO, diet containing 42 en% soybean oil.

Preparation of Lipid-Poor and Lipid-Full Serum

Rats designated for serum collection were fed one of the treatment diets for five weeks. During the mid-dark phase of the lighting cycle, unfasted rats were anesthetized lightly with ether, and blood was drawn from the exposed jugular vein. Blood from three rats of the same diet treatment was pooled and allowed to clot. The serum was drawn off through siliconized Pasteur pipettes and frozen in siliconized glass test tubes. Both lipid-full and lipid-poor serum were prepared by modification of the procedure used by Fogelman et al. (9). The serum was thawed, then heated at 56 C for 30 min, to inactivate complement. The serum was then centrifuged at 10,000 *g* for 1 hr. Next, the serum was filtered with suction through Whatman No. 40 paper, and the filtered serum was divided into two parts. Half was designated lipid-full, and the other half was delipidated (designated lipid-poor) by the method of McFarlane (10) as modified by Popjak and McCarthy (11). The filtered serum was shaken with 0.25 volumes of diethyl ether, frozen in dry ice (−70 C), then thawed slowly at room temperature. The ether layer was removed and the extracted serum stirred. The extraction was repeated four times. Any ether remaining in the serum was removed by bubbling the serum with nitrogen gas for 1 hr at 4 C. Cholesterol and triglyceride contents of the diluted lipid-full and lipid-poor sera were analyzed (Table 2) by using a Technicon Autoanalyzer II Continuous Flow instrument.

Leukocyte Isolation and Incubation

At the appropriate time in the light cycle, a rat weighing from 375 to 425 g was anesthetized lightly with diethyl ether. The intestinal cavity was opened, and blood was drawn from the aorta into a plastic syringe containing 1.0 ml of 50 mM Na_2EDTA in 50 mM K_2HPO_4, pH 6.5. Then syringes were rocked gently to mix the anticoagulant with the blood, and a sample was taken by using a Unopette capillary pipette (Becton Dickinson) for the counting of leukocytes in whole blood by using a Neubauer hemocytometer. Following the method employed by Young and Rodwell (12), leukocytes were isolated by sedimenting the red cells in a starch polymer and centrifuging the leukocyte-rich supernatant. The leukocyte pellet was resuspended in an original volume of ice-cold Krebs-Henseleit buffer (K-H) or 90.2 volume K-H without bicarbonate when the incubation was performed in presence of 4 ml lipid-full or lipid-poor serum. The final volume was 5 ml and contained 20 mM [U-^{14}C] alanine or [1-^{14}C] acetate (approximately 6 μCi each, New England Nuclear, Boston, Massachusetts). The gas phase was 95% O_2:5% CO_2, and the incubation was conducted for 360 min at 37 C, in a gyrotory water bath shaker oscillating at 110 rpm (12).

Liver Slice Preparation and Incubation

Immediately after blood was drawn, the medial lobe of the liver was excised and placed in ice-cold 0.9% NaCl. Slices of 200 ± 50 mg were cut by using a Stadie-Riggs microtome. Each slice then was incubated under conditions identical to those of the leukocyte incubation, except the duration was 120 min.

Cholesterol Incorporation Rate

[^{14}C] Cholesterol was isolated as the digitonide from the liver slices or the total leukocyte incubation medium by previously published procedures (13). Rate of incorporation was corrected for boiled blanks and known losses of label during cholesterogenesis.

Statistical Procedures

Analysis of variance and planned com-

parisons of treatment means by the t-statistic were analyzed in each experiment by use of the Statistical Package for the Social Sciences (14) and Minitab (15) computer programs.

RESULTS AND DISCUSSION

Rat Aging Studies

We have conducted three rat aging studies comparing food fats differing primarily in polyunsaturation of the fatty acids. The three studies had different objectives and designs, and the only common parameter for comparison was mortality at 15 mo (Table 3). The study published in 1972 (1) showed that minimal linoleate (1–2% of energy, en%) in the diet was more conducive to early death than linoleate (from corn oil) at about 20 en% ($P < 0.05$ for both sexes; males greater than females $P < 0.05$). In the 1978 study (2), very few of those rats fed 3 or 11% linoleate (beef tallow plus soybean oil) had died by 15 mo, but excessive linoleate (safflower oil, 30 en%) was extremely deleterious ($P < 0.001$). With advancing age beyond 15 mo, the death rate for males, with 3 en% linoleate, exceeded that of those fed 11 en% in males (2).

A later study (Dupont, J., Mathias, M. M., Connally, P. T., and Bowen, R. A., unpublished data) again showed that moderate polyunsaturation (soybean oil plus beef tallow) was more conducive to longevity than high polyunsaturation. Those rats had a definite increase in mortality when linoleate was 20 en% (from corn oil) or 27 en% (from safflower oil) (Fig. 1). The net results suggest that extremes of availability of linoleate have a propensity to contribute to increased mortality.

Tissue Bile Acids

In the 1972 study, cholesterol retention in the carcass from a tracer dose was determined 10 days after injection (1). No striking effects were

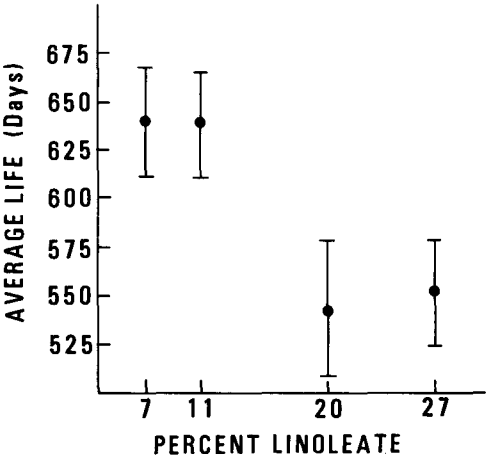

FIG. 1. Average life of male rats fed diets (details in Ref. 3) containing various concentrations of linoleate in mixtures of corn oil and beef tallow (linoleate = 7, 11 and 20 en%) or safflower oil (linoleate 27 en%).

TABLE 3

Mortality at 15 mo of Rats Fed Diets Containing Various Amounts and Kinds of Fat with Approximate Energy Content (en%) from Linoleate

		Linoleate (en%)						
		Low fat (2 en%)	High fat (40 en%)					
Study	Sex	1	2	3	7	11	20	27
		Percent dying spontaneously						
1972	M	46.2[a]	69.2[b]	—	—	—	16.7[a]	—
	F	0	36.4[b]	—	—	—	8.3[a]	—
1978	M	—	—	10.3[c]	—	7.0[c]	—	93.9[e]
	F	—	—	7.1[c]	—	0[c]	—	94.7[e]
1980	M	—	—	—	13.3[d]	6.7[d]	36.7[d]	42.1[e]

[a]Corn oil.
[b]Beef tallow + corn oil.
[c]Beef tallow + safflower oil.
[d]Beef tallow + soybean oil.
[e]Safflower oil.

seen in relation to diet, but an interesting finding was the retention of acidic steroids in nonhepatic or gastrointestinal tissues. From 15 to 43% of injected 4-^{14}C-cholesterol was recovered in an acidic lipid fraction of carcass. Additional measurements of that fraction were made in pigs and rats, and acidic steroid was found to be retained in many tissues. This led us to examine the composition of the acidic fraction. Thin layer chromatography (TLC) was the first method used, and several tissues were observed to have compounds similar to bile acids of liver and comparable to known standards (4). Samples from a number of tissues were analyzed by gas liquid chromatography (GLC) after deconjugation and derivatization to give quantitative data, and the compounds were shown by mass spectrometry to be cholanoic acids (5).

In recent years, we have repeated some of the earlier work by using high performance liquid chromatography (HPLC) (7). For these analyses, it is not necessary to deconjugate or derivatize the compounds, so the analyses are more likely to indicate the physiological state of the compound. The values for rat tissues are remarkably similar in total to those that we obtained by GLC analysis (Table 4).

In the 1978 aging study, we again measured retention of ^{14}C-cholesterol (3). Again, we saw an age effect and substantial acidic steroid in carcasses. Figure 2 illustrates the greater retention of cholesterol in older rats 28 days after a tracer dose. Figure 3 shows that, in rats 15 mo and older, 4–10% of the original dose was present in the carcass in an acidic form 28 days after administration. In the females, there was no effect of dietary linoleate concentration. In males, cholesterol retention was similar to that of females, but the higher linoleate diet resulted in less retention of acidic metabolites.

The next step was to investigate the possibility of local tissue synthesis of cholanoic acids rather than absorption from blood. We found 7α-hydroxylase activity in all the rat tissues examined (Table 5), indicating that the first necessary enzymic step was possible in the peripheral tissues.

The aim of the next experiment was to com-

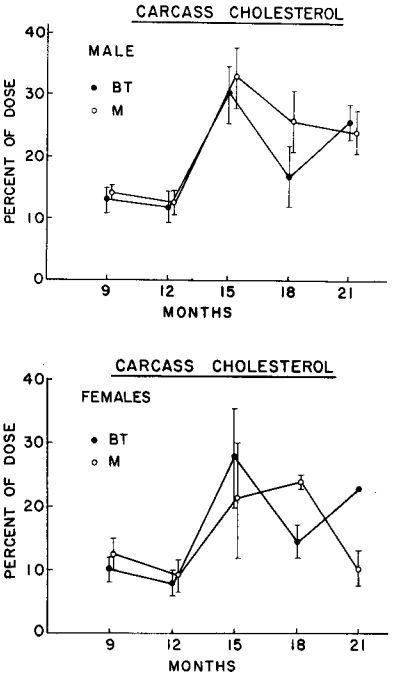

FIG. 2. Retention of 4-^{14}C-cholesterol in carcass cholesterol of aging rats (4). BT = diet containing 3% linoleate from beef tallow and safflower oil, and M = diet containing 11% linoleate from a mixture of the same fats.

TABLE 4

HPLC Quantitation of Cholanoic Acids[a] in Hepatic and Extrahepatic Tissues (7)

Tissue	Total	TC	TCDC	TDC	GC	GCDC	GDC
Rat (μg/g tissue)							
Liver	165	115	49.5	ND[b]	ND	ND	ND
Heart	13.9	9.7	4.2	ND	ND	ND	ND
Lung	11.8	4.4	7.4	ND	ND	ND	ND
Kidney	32.3	15.4	11.3	3.4	2.2	ND	ND
Adipose	5.2	2.4	3.4	2.4	ND	ND	ND
Human (μg/ml blood)							
Mixed leukocytes	1.5	0.49	0.13	ND	0.57	0.31	0.04

[a]TC, taurocholic; TDC, taurochenodeoxycholic; TDC, taurodeoxycholic; GC, glycocholic; GCDC, glycochenodeoxycholic; GDC, glycodeoxycholic acids.

[b]ND = Not detectable.

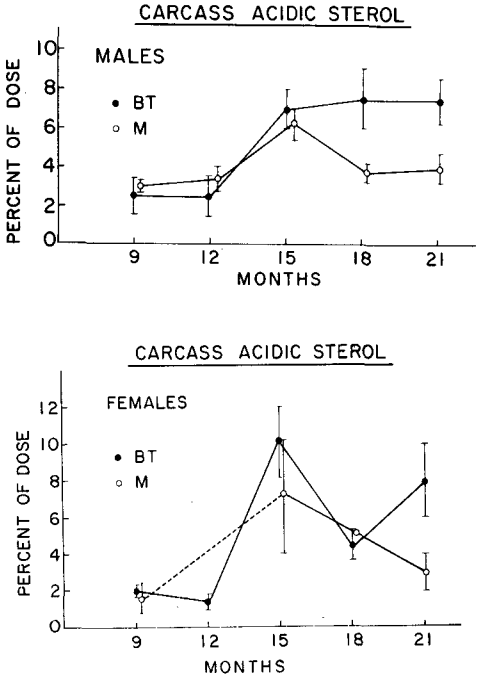

FIG. 3. Retention of 4-^{14}C-cholesterol in carcass acidic sterol of aging rats (4). BT = diet containing 3% linoleate from beef tallow and safflower oil, and M = diet containing 11% linoleate from a mixture of the same fats.

TABLE 5

Cholesterol 7α-hydroxylase Activity in Microsomes of Selected Rat Tissues (7)

Tissue	% Cholesterol conversion	nmoles/40 min
	(per mg microsomal protein)	
Liver	4.56	1.026
Heart	1.45	0.203
Lung	1.03	0.241
Kidney	1.34	0.378
Mixed leukocytes	0.247	0.113

TABLE 6

Incorporation of ^{14}C-labeled Alanine and Acetate into Cholesterol (Digitonin Precipitable Sterols) by Liver Slices and Leukocytes Taken from Rats Killed During the Mid-dark Phase (Means ±SEM)

Substrate	Liver (n = 8) (pm/min.mg)	Leukocyte (n = 5) (pm/min.10^{6}cells)
[U-^{14}C] alanine	1.32 ± .63	9.6 ± 1.4
[1-^{14}C] acetate	0.60 ± .09	5.5 ± 0.8

pare rat liver and leukocytes in regard to regulation of cholesterol synthesis. Preferred substrate, diurnal cycle, and effects of fasting, composition of dietary fats, and presence of lipid-full and lipid-poor serum were assessed. The ultimate goal was to characterize the regulation of leukocyte cholesterol metabolism to determine if it could be utilized to model human cholesterol metabolism in peripheral tissues.

Rat Leukocyte Study

Substrate preference. Table 6 illustrates the substrate incorporation rate into cholesterol (digitonin precipitable sterols) by rat liver and leukocytes. Both hepatic and leukocyte alanine incorporation exceeded acetate incorporation twofold. Results of this experiment advised use of alanine as the substrate of choice for the remaining physiological and dietary experiments.

Physiological variables. The rates of hepatic incorporation of alanine into cholesterol in response to lighting cycle and fasting are illustrated in Figure 4. Rats killed during the light phase demonstrated a 79% decrease in incorporation rate ($P < 0.005$) into cholesterol when compared with the dark-phase controls.

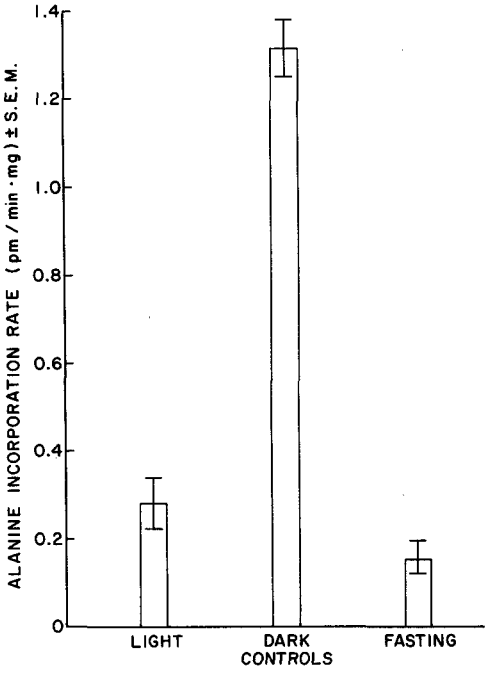

FIG. 4. Alanine incorporation into digitonin precipitable sterols by rat liver slices in response to light cycling and a 24-hr fast. Mean ±SEM (n = 8).

When rats were subjected to a 24-hr fast, substrate incorporation into cholesterol dropped significantly ($P < 0.005$) compared with the dark-phase controls (88%). Figure 5 shows the alanine incorporation by the leukocyte into cholesterol in response to light cycle and fasting. There were no significant differences ($P > 0.25$) in incorporation rates found for either physiological variable.

Serum and dietary incubation variables. The cholesterol and triglyceride contents of the lipid-full and lipid-poor sera, as used in the tissue incubations, are listed in Table 2. In the lipid-full sera, BT + CHOL had the highest cholesterol concentration (72 mg/dl), while the other sera contained the same amounts of cholesterol (55–58 mg/dl). The cholesterol concentration in the four lipid-poor sera were approximately equal (22–28 mg/dl). The triglyceride concentration averaged 74 mg/dl, and 60% was removed during preparation of the lipid-poor sera.

Figure 6 illustrates the effect of sera and dietary lipids on alanine incorporation into cholesterol by liver slices. In both the highly saturated fat (BT) and highly polyunsaturated fat (SO) diet groups, incubations of liver slices in lipid-full serum led to higher rates of incorporation than incubation in lipid-poor serum. Because only label appearing in hepatic sterols and not in the medium was determined, one explanation for these findings is that the lipid-poor serum enhanced release of cholesterol from the lipid slice into the medium. Dietary CHOL depressed hepatic cholesterol genesis as reflected by highly significant contrasts ($P < 0.001$) between groups incubated in lipid-full serum. There were no significant effects on the rate of incorporation by the saturation of fat in the diet.

Figure 7 illustrates results obtained from incubation of leukocytes under analogous variables, but in this case total sterol synthesis was assessed. There was a consistent pattern of increased ($P < 0.002$) alanine incorporation into cholesterol due to the presence of lipid-poor compared with lipid-full sera. This result is consistent with the known effects of feedback regulation of cholesterol synthesis. There was no effect of cholesterol feeding or saturation of fat on incorporation rates ($P > 0.25$).

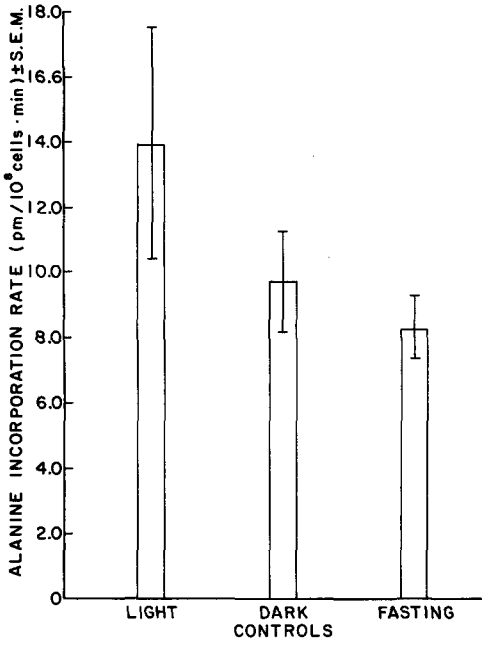

FIG. 5. Alanine incorporation into digitonin precipitable sterols by rat leukocytes in response to light cycling and 24-hr fast. Mean ±SEM (n = 5).

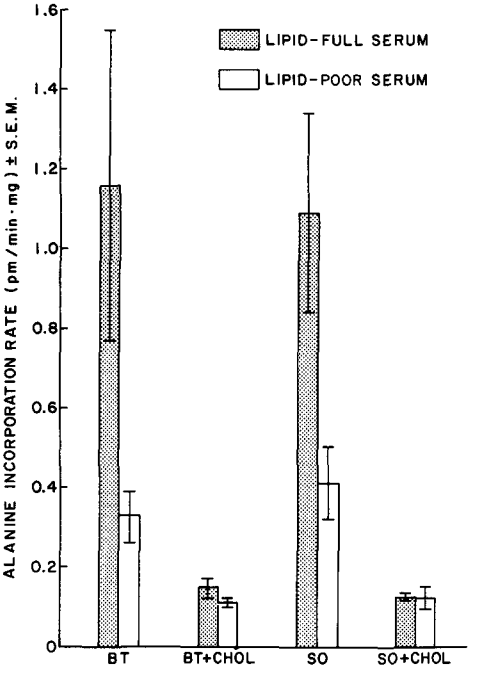

FIG. 6. Alanine incorporation into digitonin precipitable sterols of liver slices from rats of the four experimental diet groups. Liver slices were incubated for 2 hr in lipid-full and lipid-poor serum. Mean ±SEM (n = 8). BT, diet containing 19% by weight of beef tallow; CHOL, 3% by weight of cholesterol; SO, diet containing 19% by weight of safflower oil.

Human Studies

From the results with rat leukocytes, we concluded that leukocytes were more like peripheral tissues than like liver, and so chose them to study as a model for regulation of cholesterol metabolism in the human. We studied groups of healthy males of different ages (7). Their leukocytes contained bile acids (Table 7) and hydroxymethylglutaryl coenzyme A reductase (HMGR) and 7α-hydroxylase (COH) activities (Table 8). They also had bile acids in each lipoprotein fraction (Table 9). The men consumed similar diets (P/S = 0.4, cholesterol 300–400 mg/day) at all ages, so there were no dietary correlations. There were correlations of age with cholesterol metabolizing enzymes. Both enzyme activities increased with age and with LDL concentration. This finding in relation to LDL is contrary to the observations made in rats by using lipid-full compared with lipid-poor serum. Based upon present knowledge of mechanisms of LDL cholesterol metabolism, it seems that the rat leukocytes responded to decreased cholesterol by increasing their cholesterol synthesis. On the other hand, there may be a decrease in receptor activity in the human leukocytes with advancing age and, therefore, a failure in inhibition of cholesterol synthetic activity.

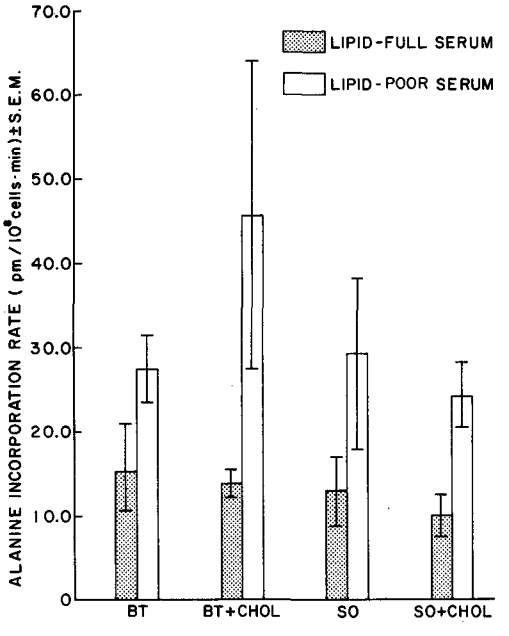

FIG. 7. Alanine incorporation into digitonin precipitable sterols by leukocytes from rats of the four experimental diet groups. Leukocytes were incubated for 2 hr in lipid-full and lipid-poor serum. Mean ±SEM (n = 8). BT, diet containing 19% by weight of beef tallow; CHOL, 3% by weight of cholesterol; SO, diet containing 19% by weight of safflower oil.

CONCLUSIONS

A dietary content of 3 to 11 en% of linoleate seems to be most favorable for longevity of rats, with males more susceptible than females to deleterious effects of lower levels.

Various reasons for differences in life span related to dietary fat have been discussed by others. Harmon (16,17) has studied and reviewed the interactions of polyunsaturated fatty acids and antioxidants in the diet. All the diets used in our studies had adequate quantities of α-tocopherol, so excessive free-radical

TABLE 7

Total and Differential Leukocyte Count and Cholesterol and Bile Acid Concentrations in Leukocytes of Healthy Men in Three Different Age Groups (Mean ±SEM) (7)

Variable	Age groups, yr		
	19–25 (n = 10)	40–50 (n = 9)	60–70 (n = 10)
Counts			
Total number/ml plasma	7194 ± 474	6494 ± 417	7816 ± 694
Leukocytes (%)	44 ± 2.7	40 ± 3.4	38 ± 2.9
Granulocytes (%)	56 ± 2.7	60 ± 3.4	62 ± 2.9
Cholesterol (μg per 10^8 cells)			
Total	101 ± 11.9	106 ± 14.3	135 ± 28.1[a]
Free cholesterol	78 ± 10.3	79 ± 10.8	100 ± 18.9[a]
Cholesteryl ester	23 ± 3.1	27 ± 3.8	35 ± 9.4
Bile acids (nmoles per 10^8 cells)	2.11 ± 0.16	2.16 ± 0.25	2.11 ± 0.18

[a]Significantly different ($P < 0.05$).

TABLE 8

Hydroxymethylglutaryl Coenzyme A Reductase (HMGR)[a] and Cholesterol 7α-hydroxylase (COH)[b] Activity in Mononuclear Leukocyte Microsomes of Healthy Men in Three Different Age Groups (Mean ±SEM) (7)

Enzyme activity expressed on basis of:	Age groups, yr[c]					
	19-25 (n = 10)		40-50 (n = 9)		60-70 (n = 10)	
	HMGR	COH	HMGR	COH	HMGR	COH
Per mg microsomal protein	10.5[a] ± 0.54	0.106[a] ± 0.0059	13.1[b] ± 0.81	0.107[a] ± 0.0051	14.4[b] ± 1.22	0.128[a] ± 0.0113
Per 10^7 mononuclear cells	30.4[a] ± 3.24	0.303[a] ± 0.0300	51.4[b] ± 6.52	0.419[ab] ± 0.0549	45.2[b] ± 4.10	0.401[b] ± 0.0386
Per ml blood	9.1[a] ± 0.49	0.091[a] ± 0.0054	12.2[b] ± 0.75	0.099[a] ± 0.0079	12.5[b] ± 0.77	0.110[a] ± 0.0069

[a]Enzyme activity was expressed as pmoles of mevalonate formed per hr.

[b]Enzyme activity was expressed as % of ^{14}C-cholesterol converted to ^{14}C-7α-hydroxycholesterol per 40 min.

[c]Means for each age group with different letter superscripts within the same row are significantly different ($P < 0.05$).

TABLE 9

Bile Acids[a] in Human Plasma Lipoproteins (7)

Lipoprtein	μg/dl	% of total steroid
VLDL	47.5	0.21
LDL	177.4	0.11
HDL	93.6	0.20

[a]Calculated on the basis of glycoholic acid mass.

formation does not seem to account for differences in longevity. Birt et al. (18) reported longer life span of Syrian hamsters when approximately 20 en% linoleate was fed compared with 10 or 5 en% (from corn oil). Other components of the diet also were varied. Possible involvement of eicosanoids has been discussed (2) and reviewed (19). A range from 2 to 15 en% linoleate should be tested more closely, but outside that range, combinations with other factors may result in reduction of life span, depending on other components of the diet.

We still do not understand the mechanisms of effects of dietary fat upon cholesterol metabolism. The regulation of cholesterol metabolism includes events in peripheral tissues that may include conversion of cholesterol to acidic steroids. Use of the leukocyte offers a good biopsy tissue for human studies. Responses of the young rat leukocyte to lipids in serum may be analogous to young humans. The decrease in ability of older men to respond to plasma cholesterol concentration may be an important mechanism for the rise in serum cholesterol with age (1,2,20,21).

The participation of bile acids in regulation of cholesterol metabolism in lipoproteins and peripheral tissues requires additional exploration. The coordination of HMGR and COH activities in leukocytes suggests a function for bile acids that could be analogous to its functions with cholesterol esterification enzymes (22,23).

ACKNOWLEDGMENTS

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Pulmonary Surfactant Lipid Synthesis from Ketone Bodies, Lactate and Glucose in Newborn Rats

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ABSTRACT

The contribution of acetoacetate (AcAc), β-hydroxybutyrate (βOHB), lactate and glucose to pulmonary surfactant lipid synthesis in three- to five-day-old rats was measured. Minced lung tissue was incubated with ^3H_2O and [3-^{14}C]AcAc, [3-^{14}C]βOHB, [U-^{14}C]lactate or [U-^{14}C]glucose, and the radioactivity incorporated into surfactant lipids was measured. When expressed as nmol of substrate incorporated/g lung tissue per four hr, lactate was incorporated more rapidly than other substrates into total surfactant lipids and phosphatidylcholine (PC). There was no difference in the rates of incorporation of lactate, AcAc or glucose into disaturated PC (DSPC). Substrates other than glucose were incorporated almost exclusively into fatty acids, whereas 60–80% of glucose incorporated into surfactant phospholipids was found in fatty acids, with the remaining in glyceride-glycerol. When expressed as nmol acetyl units incorporated/g lung tissue per four hr, the rates of AcAc, lactate and glucose incorporation into total surfactant fatty acids were comparable. Glucose incorporation into DSPC and PC was greater than that of AcAc and lactate. When glucose was the only exogenous substrate added to the incubation medium, it contributed 37% of total surfactant fatty acids synthesized de novo. In the presence of other substrates, the contribution of glucose to de novo fatty acid synthesis dropped to 14–20%. In the presence of unlabeled glucose, ^{14}C-labeled AcAc, lactate and βOHB contributed 52%, 40% and 19%, respectively, of the total fatty acids synthesized de novo. The rate of βOHB incorporation into surfactant lipids was only about 50% that of other substrates and was accompanied by low activity of β-hydroxybutyrate dehydrogenase measured for newborn lung. These results demonstrate that AcAc and lactate are important precursors for surfactant lipids in neonatal rat lung.

Lipids 20:835–841, 1985.

INTRODUCTION

De novo synthesis of phospholipids and their incorporation into pulmonary surfactant are essential in maintaining normal lung function (1). The availability of fatty acids, rate-limiting in phospholipid biosynthesis (2,3), can be derived from de novo synthesis or from the circulation (4–6). Although utilization of circulating fatty acids for complex lipid synthesis may be enhanced under certain conditions, such as the increase in plasma-free fatty acids during the suckling period (4), fatty acids synthesized de novo also are important for synthesis of surfactant phospholipids, particularly DSPC (6,7). Buechler and Rhoades (8) reported that ca. 80% of the fatty acids synthesized de novo in the lung were incorporated into PC, 91% of which was present in DSPC. It has been determined that 40% of all fatty acids esterified to lung DSPC are of de novo origin (6).

It generally is accepted that glucose is the major physiologic precursor of fatty acids in adult lung (9,10). In vitro studies showed that the rate of glucose incorporation into lipids in adult lung was at least twice as high as that in neonatal lung (4), suggesting a greater reliance on substrates other than glucose for de novo fatty acid synthesis during perinatal stages. Lactate, pyruvate, acetate and amino acids can be incorporated into phospholipids in lung and hence could serve as alternative sources (8,10,11).

We recently have demonstrated that AcAc is incorporated rapidly into lung fatty acids in vivo and in vitro (12–14). In the present experiments, we investigated the contributions of AcAc, βOHB, lactate and glucose to surfactant lipid biosynthesis in newborn rats.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats were raised by breeding procedures described previously (15). Three to five-day-old rats were used, because pulmonary fatty acid synthesis is higher at this age than in older suckling rats (13).

Metabolic Studies

Rats were anesthetized with sodium pentobarbital (5 mg/100 g body weight) and lungs were removed, dissected free of vascular tissue

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and minced for metabolic studies as previously described (14). The incubation system, in a final volume of 2 ml, consisted of Ca^{2+}-free Krebs-Ringer bicarbonate buffer, pH 7.4; 33 mg bovine serum albumin; 1 mCi ^3H_2O; and 20 μmol of [3-^{14}C]AcAc, [U-^{14}C]lactate or [U-^{14}C]glucose, or 10 μmol [3-^{14}C]D-βOHB containing 1 μCi radioactivity. Linear rates of lipid synthesis were obtained with 2–20 mM ketone bodies and 5–20 mM glucose and lactate. Incubations of ^{14}C-labeled ketone bodies and lactate contained 20 μmol cold glucose to stimulate maximal activity (14).

Incubation conditions were as described (13) except that incubation time was four hr. Substrate incorporation into surfactant chloroform/methanol extractable lipids and phosphatidylcholine was linear for at least six hr. After incubation, the reaction was stopped by placing the flasks on ice. Two to four samples were pooled and homogenized using a Ten-Broek homogenizer, and surfactant was isolated by the method of Frosolono et al. (16) as modified by Sanders and Longmore (17). It should be emphasized that although surfactant is thought to originate from type II pneumocytes, the surfactant fraction isolated by this method actually represents material derived from the intracellular and extracellular pools (17).

Lipid Analysis

Lipids were extracted from surfactant in chloroform/methanol (2:1, v/v) by the method of Folch et al. (18) and evaporated to dryness under nitrogen. One aliquot was saponified as described previously (14), and radioactivity was determined in total lipid, fatty acids, nonsaponifiable lipid and glyceride-glycerol. An aliquot was taken for separation of PC, other phospholipids, and neutral lipids by two-dimensional thin layer chromatography (TLC) on silica gel H, 250 μm thick. The solvent system in the first dimension was chloroform/methanol/7 M NH_4OH (65:35:5, v/v/v), and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5, v/v/v/v/v) in the second dimension. Lipids were visualized with iodine vapors and identified by comparison with known standards. PC and the pooled fraction of other phospholipid were scraped, and the lipids were extracted from silica gel with three 5 ml aliquots of chloroform/methanol (1:1, v/v) containing 10% water. Lipid extracts were dried under nitrogen and saponified. The radioactivity in the total lipid, fatty acid and glyceride-glycerol fractions was measured (14).

DSPC was isolated from PC by the method of Mason et al. (19). After reaction with osmium tetroxide, DSPC was separated from unsaturated phosphatidylcholine (USPC) by TLC on silica gel H using chloroform/methanol/7 M NH_4OH (80:28:6, v/v/v). USPC and DSPC were scraped, extracted and saponified, and the radioactivity in each fraction was determined as described above.

Radioactivity of lipid fractions was determined by liquid scintillation counter (Beckman Model LS6800, Houston, Texas). Lipid samples were counted in a toluene scintillation fluid (14) and glyceride-glycerol samples were counted in ACS (Amersham, Arlington Heights, Illinois).

Incorporation of ^3H_2O into fatty acids and glyceride-glycerol was expressed as acetyl groups by multiplying nmol substrate incorporated by 1.15 and 0.91, respectively (20). For ^{14}C-labeled substrates, the acetyl units were calculated on the basis of 3 nmol acetyl units/nmol glucose, 2 nmol acetyl units/nmol AcAc or βOHB and 1.5 nmol acetyl units/nmol lactate (4). Similar calculations were made for glucose incorporation into glyceride-glycerol. The rates of lipogenesis therefore were expressed as nmol substrate incorporated or nmol acetyl unit incorporated into surfactant isolated per g of lung tissue per four hr.

βOHB Dehydrogenase Assay

D(−)-β-Hydroxybutyrate dehydrogenase (EC 1.1.1.30) activity of lung, brain and liver homogenates of three- to five-day-old rats was assayed by the method of Williamson et al. (21).

Chemicals

Radioactive substrates were purchased from New England Nuclear (Boston, Massachusetts). Ethyl[3-^{14}C]AcAc was converted to [3-^{14}C]AcAc by the method of Krebs and Eggleston (22). Lipid standards were from Supelco (Bellefonte, Pennsylvania). Organic solvents were from Fisher Scientific Co. (Pittsburgh, Pennsylvania) and were redistilled before use. Bovine serum albumin (Fraction V), substrates and other chemicals were purchased from Sigma Chemical Co. (St. Louis, Missouri).

Statistical Analysis

Data were analyzed by one-way analysis of variance. Comparisons of treatment means were made by Duncan's New Multiple Range Test (23).

RESULTS

The greatest rate of substrate incorporation into surfactant chloroform/methanol extract-

able lipids was observed with lactate (Table 1). AcAc and glucose both were used at 80% of the rate of lactate, whereas βOHB incorporation was only 40–50% of that of the other substrates. Lactate also was the most active substrate for surfactant fatty acid synthesis. The rate of AcAc incorporation into surfactant fatty acids was 1.5× that of glucose and twice that of βOHB. There was little incorporation of substrates into nonsaponifiable lipid. Ketone bodies and lactate were used almost exclusively for fatty acid synthesis, while 38% of the glucose incorporated into surfactant lipid was in the glyceride-glycerol fraction.

The pattern of substrate incorporation into surfactant PC parallels that into total lipids (Table 2). Lactate was incorporated into total PC and PC fatty acids at a greater rate than were the other substrates. There was no difference in glucose and AcAc incorporation into total PC; however, AcAc was incorporated at a greater rate than glucose into PC fatty acids. Surfactant PC and PC fatty acid synthesis from βOHB was only 50% that from AcAc and 38% that from lactate. The extent of glucose incorporation into PC glyceride-glycerol (34%) was similar to that for total surfactant lipids.

The incorporation of each substrate into surfactant phospholipids other than PC also was measured and a pattern of incorporation similar to that for PC was observed (data not shown). Regardless of the substrate, 84–89% of surfactant phospholipid radioactivity was found in the PC fraction.

In contrast to total surfactant lipids and surfactant PC, there was no difference in incorporation of lactate, AcAc or glucose into total DSPC (Table 2). The rate of βOHB utilization for DSPC synthesis was only 45–56% of that for the other substrates, a finding similar to those for total surfactant lipids and PC. DSPC fatty acid synthesis from lactate was greater than from glucose or AcAc, which had equivalent rates of incorporation. β-Hydroxybutyrate was utilized for DSPC fatty acid synthesis at a rate only 45–62% that of AcAc, lactate and glucose. Twenty-four percent of glucose used in DSPC synthesis was present in the glyceride-glycerol fraction. The patterns of substrate incorporation into USPC were similar to those for DSPC (data not shown). Regardless

TABLE 1

Incorporation of ^{14}C-Labeled Substrates into Surfactant Lipids in Neonatal Rat Lung

	nmol substrate incorporated/g lung tissue per 4 hr			
Substrate	Total lipid	Fatty acids	Nonsaponifiable lipid	Glyceride-glycerol
[3-^{14}C]Acetoacetate	160 ± 9^a	138 ± 7^a	5 ± 0.3^a	—
[3-^{14}C]D-β-Hydroxybutyrate	83 ± 6^b	67 ± 5^b	2 ± 0.4^b	—
[U-^{14}C]Glucose	166 ± 6^a	85 ± 6^c	2 ± 0.2^b	63 ± 3
[U-^{14}C]Lactate	200 ± 8^c	157 ± 5^d	4 ± 0.4^a	—

Values are means ± SEM for eight or nine samples. Fatty acids represent a sum of esterified and free fatty acids. All values within a column not sharing a common superscript are significantly different ($P < 0.05$).

TABLE 2

Incorporation of ^{14}C-Labeled Substrates into Surfactant Phosphatidylcholine in Neonatal Rat Lung

	nmol substrate incorporated/g lung tissue per 4 hr					
	Phosphatidylcholine			Disaturated phosphatidylcholine		
Substrate	Total PC	Fatty acids	Glyceride-glycerol	Total DSPC	Fatty acids	Glyceride-glycerol
[3-^{14}C]Acetoacetate	105 ± 4^a	97 ± 4^a	—	70 ± 3^a	61 ± 2^a	—
[3-^{14}C]D-β-Hydroxybutyrate	52 ± 6^b	49 ± 3^b	—	39 ± 4^b	38 ± 3^b	—
[U-^{14}C]Glucose	116 ± 5^a	76 ± 4^c	40 ± 3	83 ± 8^a	61 ± 5^a	20 ± 1
[U-^{14}C]Lactate	141 ± 5^c	127 ± 5^d	—	86 ± 6^a	77 ± 4^c	—

Values are means ± SEM for eight or nine samples. All values within a column not sharing a common superscript are significantly different ($P < 0.05$). PC, phosphatidylcholine; DSPC, disaturated phosphatidylcholine.

of the substrate, 74–77% was incorporated into DSPC and 23–26% into USPC.

For direct comparison of different substrates as carbon sources of surfactant lipids, the rate of substrate incorporation into lipids was calculated on the basis of acetyl groups as described in the Materials and Methods section. When expressed as nmol acetyl units incorporated/g lung tissue per four hr, the rates of AcAc, glucose and lactate incorporation into total surfactant fatty acids were comparable (Table 3). In contrast, glucose incorporation into PC and DSPC fatty acids was greater than that of AcAc and lactate. There was no difference in incorporation between AcAc and lactate. Incorporation of AcAc and lactate into PC fatty acids was about 85% of the rate for glucose and 70% of the rate for glucose for DSPC fatty acid synthesis. Among all substrates, βOHB had the lowest rates of acetyl unit incorporation into all lipid products investigated.

The contribution of acetyl units derived from each substrate to total de novo fatty acid synthesis is presented in Figure 1. Total fatty acids synthesized de novo were estimated by converting tritium incorporation from ^3H_2O into acetyl units, as described in the Materials and Methods section. Percent contribution by ^{14}C-labeled substrates was calculated by using the total synthesis estimated for each experiment (Fig. 1A and B). Glucose provided 37% of the total de novo fatty acids incorporated into surfactant when it was the only substrate in the incubation. The contribution of glucose to total de novo fatty acid synthesis was decreased to 14, 15 and 20% in the presence of lactate, AcAc and βOHB, respectively (Fig. 1A). The presence of glucose in the incubation medium facilitated incorporation of other substrates into fatty acids (data not shown). AcAc contributed 52% of the total fatty acids synthesized de novo despite the presence of glucose in the medium (Fig. 1B). Under the same incubation conditions, lactate and βOHB contributed 40% and 19%, respectively, of the total fatty acids synthesized.

The rate of de novo fatty acid synthesis estimated from ^3H_2O incorporation in the presence of AcAc or lactate was 13–27% less than in the presence of glucose or βOHB. The reason for this difference is unknown. This inhibitory effect was not observed in experiments in which tissue lipids, rather than surfactant lipids, were examined.

To determine the contribution of glucose to total de novo glyceride-glycerol synthesis, [^{14}C]glucose and ^3H_2O incorporation into glyceride-glycerol were converted to acetyl units, as described in the Materials and Methods section. With glucose as the only carbon source available, 86% of glyceride-glycerol synthesized was from glucose (Table 4). In the presence of AcAc, lactate or βOHB, glucose also provided 82–92% of glyceride-glycerol synthesized.

In the present experiments with pulmonary surfactant lipids and previous experiments with whole lung lipids (14), we found that the rate of βOHB utilization for lipogenesis was con-

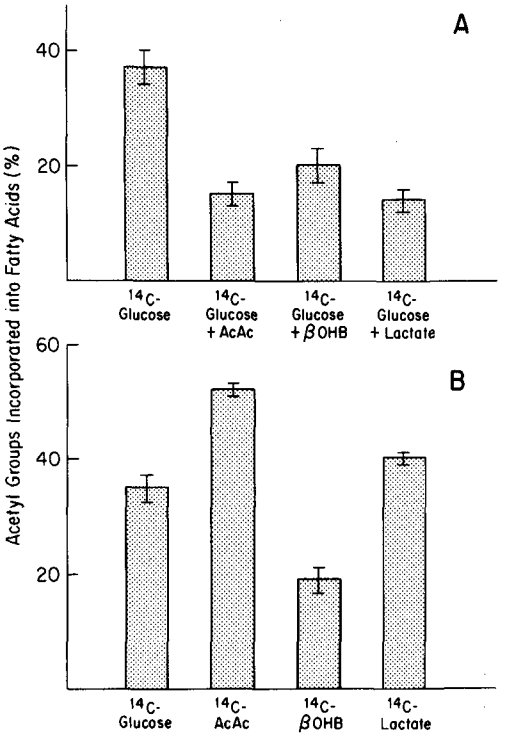

FIG. 1. A. Percent contribution of [U-^{14}C]glucose in the presence of unlabeled substrates to total surfactant fatty acid synthesis. Minced lung tissue from three- to five-day-old rats was incubated with 20 nmol [U-^{14}C]glucose (1 μCi), 1 mCi ^3H_2O in the presence or absence of unlabeled AcAc, βOHB or lactate. Surfactant fatty acids were isolated, and acetyl units derived from glucose and ^3H_2O were calculated as described in Materials and Methods. Total surfactant fatty acid synthesis (100%) was 857 ± 47, 748 ± 42, 924 ± 62 and 690 ± 8 nmol acetyl units incorporated/g tissue per four hr for incubations of [U-^{14}C]glucose, [U-^{14}C]glucose + AcAc, [U-^{14}C]glucose + βOHB and [U-^{14}C]glucose + lactate, respectively. B. Percent contribution of ^{14}C-labeled substrates to total surfactant fatty acid synthesis. Total surfactant synthesis (100%) was 724 ± 28, 526 ± 32, 734 ± 60 and 594 ± 28 acetyl units incorporated/g tissue per four hr for incubations of [U-^{14}C]glucose, [3-^{14}C]AcAc + glucose, [3-^{14}C]βHOB + glucose and [U-^{14}C]lactate + glucose, respectively.

TABLE 3

Quantitative Comparison of ^{14}C-Labeled Substrate Incorporation into Surfactant Fatty Acids

Substrate	nmol acetyl units incorporated/g lung tissue per 4 hr		
	Total fatty acids	PC fatty acids	DSPC fatty acids
[3-^{14}C]Acetoacetate	275 ± 15[a]	194 ± 8[a]	122 ± 4[a]
[3-^{14}C]D-β-Hydroxybutyrate	134 ± 10[b]	98 ± 6[b]	76 ± 6[b]
[U-^{14}C]Glucose	254 ± 19[a]	228 ± 12[c]	177 ± 15[c]
[U-^{14}C]Lactate	235 ± 8[a]	191 ± 7[a]	118 ± 7[a]

Values are means ± SEM for eight or nine samples. All values within a column not sharing a common superscript are significantly different ($P < 0.05$). PC, phosphatidylcholine; DSPC, disaturated phosphatidylcholine.

TABLE 4

Contribution of Glucose to Total Surfactant Lipid Glyceride-Glycerol Synthesis in Neonatal Rat Lung

Substrate	nmol acetyl units incorporated/g lung tissue per 4 hr		
	Acetyl unit equivalents		
	Derived from glucose	Derived from ^3H_2O	% of Total derived from glucose
[U-^{14}C]Glucose	179 ± 11[a,b]	207 ± 4[a,b]	86.2 ± 4.2[a]
[U-^{14}C]Glucose + AcAc	145 ± 2[c]	174 ± 4[c]	83.4 ± 1.4[a]
[U-^{14}C]Glucose + βOHB	203 ± 16[a]	221 ± 16[a]	92.1 ± 4.4[a]
[U-^{14}C]Glucose + lactate	157 ± 4[b,c]	191 ± 10[b,c]	82.5 + 3.4[a]

Values are means ± SEM for four or five samples. All values within a column not sharing a common superscript are significantly different ($P < 0.05$).

sistently low, about 50% that of other substrates tested. To investigate possible mechanism(s) for the poor βOHB utilization in newborn lung, activity of βOHB dehydrogenase, the first step in βOHB metabolism, was measured in lung of three- to five-day-old rats (Table 5). For comparison, βOHB dehydrogenase activity also was measured in liver and brain of rats the same age. In lung homogenates, βOHB dehydrogenase activity was only 19% and 0.8% of the activities in brain and liver, respectively, when expressed on a tissue weight basis. Based on mg of tissue protein, βOHB dehydrogenase activity of lung was only 12 and 1.3% of activity of brain and liver, respectively.

TABLE 5

Activity of D(−)-β-hydroxybutyric Dehydrogenase in Lung, Brain and Liver of Neonatal Rats

Tissue	βOHB dehydrogenase activity	
	nmol AcAc formed/ g tissue per min	nmol AcAc formed/ mg protein per min
Lung	45 ± 2[a]	0.44 ± 0.06[a]
Brain	239 ± 7[b]	3.53 ± 0.08[b]
Liver	5595 ± 296[c]	32.61 ± 2.14[c]

Values are means ± SEM of four to six samples. All values within a column not sharing a common superscript are significantly different ($P < 0.05$). βOHB, D(−)-β-hydroxybutyrate.

DISCUSSION

Pulmonary surfactant is synthesized and secreted into the alveoli by the type II pneumocytes. Because surfactant is synthesized by only one of the 40 cell types estimated to be present in the lung, comprising only 14% of the total cell mass (24), studies of lipid metabolism of whole lung homogenates have shed little light on the metabolism of pulmonary surfactant. One approach to this problem has been study of the isolated type II pneumocyte. Metabolism of isolated type II pneumocytes may not reflect that of the type II pneumocyte within intact tissue, because of the isolation procedure itself and the artificial

environment of cell culture (25,26). We have developed a model for studying pulmonary surfactant metabolism that uses ^{14}C-labeled substrates incubated with minced lung tissue, with subsequent isolation and analysis of pulmonary surfactant. One advantage of this system is that all pulmonary cell types are present during the incubation, more closely resembling in vivo conditions. In addition, freshly excised tissue is used. These considerations, together with the potential for utilization of substrates other than glucose for lung lipid synthesis (4,8,10,11,13) prompted us to investigate the incorporation of AcAc, βOHB, lactate and glucose into pulmonary surfactant, isolated after incubation, of three- to five-day-old rats.

The results show that lactate, AcAc and glucose incubated with minced lung are incorporated readily into surfactant lipids, particularly PC and DSPC. Substrates other than glucose were incorporated almost exclusively into the fatty acid moiety of complex lipids. Although glucose predominantly was found in fatty acids, about 20–40% of the carbons recovered in phospholipids were in glyceride-glycerol. Taken together, these results indicate that lactate, AcAc and glucose are alternative substrates for synthesis of surfactant lipids, particularly PC. This is substantiated further by findings that, expressed as acetyl groups, the contribution of AcAc and lactate to the fatty acid moiety of PC and DSPC was 67–85% that of glucose. Furthermore, carbon contribution from AcAc, lactate and glucose for fatty acids of total surfactant lipid was the same. It is noteworthy that lactate also is utilized as rapidly as glucose for fatty acid synthesis in type II pneumocytes (5).

Consistent with our earlier study using lung homogenate (14), the rate of βOHB incorporation into surfactant lipids was about 50–60% that of AcAc. The reason for the depressed rate of βOHB incorporation into various lipids is not clear. Compared to brain and liver of developing rats, the activity in lung tissue was extremely low, less than 20% of brain activity and less than 1.5% of liver activity. β-Hydroxybutyrate dehydrogenase is the first and possibly rate-limiting step of βOHB metabolism (27,28). Although the enzyme activity measured in lung tissue may not reflect true activity in type II pneumocyte, the low activity appeared to be associated with the low rate of βOHB incorporation into surfactant lipids determined from the minced tissue. Moreover, the more rapid rate of AcAc, compared to βOHB, incorporation into fatty acids may be attributed to our earlier finding that AcAc is metabolized almost exclusively by the acetoacetyl CoA synthetase (EC 6.2.1.X) and acetoacetyl CoA thiolase (EC 2.3.1.9) pathway, thereby bypassing mitochondrial involvement for acetyl CoA production (14).

The use of tritium incorporation into fatty acids to estimate total de novo fatty acid synthesis has been applied widely to lung tissue (4–6,8). Using ^{3}H of tritiated water in combination with ^{14}C-labeled substrates, we determined the contribution of each ^{14}C-labeled substrate to total de novo surfactant fatty acid synthesis under the experimental conditions. When glucose was the only exogenous substrate in the incubation, it contributed about 40% of total fatty acids synthesized de novo. The contribution by glucose was reduced markedly in the presence of AcAc, βOHB and lactate. Although the inhibition mechanism is not understood fully, it is likely that acetyl CoA from these metabolites dilutes the acetyl CoA pool derived from glucose. Conversely, addition of glucose to the incubation medium stimulated the rates of fatty acid synthesis from ^{14}C-labeled lactate, AcAc of βOHB (data not shown). These observations suggest that glucose serves not only as a major source of acetyl units for fatty acid synthesis but also facilitates lipogenesis from other substrates by providing glycerol-3-phosphate and reducing equivalents (2,5). Lactate, AcAc and glucose are important precursors of surfactant lipids, but their relative contribution to overall de novo synthesis must depend upon concentrations of the individual substrates presented to type II pneumocytes.

The physiological situation undoubtedly is more complex than that represented by our in vitro conditions. Several potential substrates for fatty acid synthesis are present in the cell simultaneously at levels that vary with the age and physiological state of the animal. For example, plasma levels of ketone bodies are elevated and glucose levels are depressed in the neonate (29,30). Hormonal influences also may alter the flux through any of the pathways of fatty acid synthesis from precursors. Experiments such as these cannot address directly the question of the contribution of glucose, AcAc, lactate or βOHB to de novo surfactant fatty acid synthesis in vivo; nonetheless, it is clear from the present experiments that there is significant potential for the synthesis of surfactant fatty acids from AcAc and lactate.

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Lipid Composition and Prostaglandin Synthesis in Mouse Lung Microsomes: Alterations Following the Ingestion of Menhaden Oil

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ABSTRACT

Three groups of male mice were fed a normal diet or a semisynthetic diet containing either 10% hydrogenated coconut oil (CO group) or 10% menhaden oil (MO group) for two wk. The synthetic diet altered the fatty acid composition of lung microsomal lipids. Mice ingesting menhaden oil contained greater amounts of eicosapentaenoic acid (20:5 n-3), docosapentaenoic acid (22:5 n-3) and docosahexaenoic acids (22:6 n-3) and decreased amounts of n-6 fatty acids such as arachidonic and adrenic. Synthesis of prostaglandin E_2 and prostaglandin $F_{2\alpha}$ from exogenous arachidonic acid was significantly depressed in n-3 fatty acid-enriched lung microsomes. These studies indicated that dietary fish oil not only alters the fatty acid composition of lung microsomes but also lowers the capacity of lungs to synthesize prostaglandins from arachidonic acid.

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INTRODUCTION

The synthesis of prostaglandins, hydroxy fatty acids and leukotrienes by lungs has been studied in some detail in recent years (1-3). These eicosanoids have profound physiological, biochemical and immunological effects, suggesting that lungs may play an important role in many biological functions mediated by eicosanoids (1,4,5). Prostaglandin synthesis in lungs is affected by several factors, including drugs (6), antioxidants (7), radiation (8) and cigarette smoke (9). There is also marked variation in the type and quantity of prostaglandins synthesized in lungs of different species (10,11). We demonstrated recently that dietary n-3 fatty acids from fish oil depressed thromboxane, prostaglandin E_2 and prostaglandin $F_{2\alpha}$ synthesis in rats and guinea pigs, with the former being most affected (11).

There is considerable interest in the putative beneficial effects of fish oil on cardiovascular disease, hypertension, atherosclerosis and cancer (12-15). These beneficial effects have been attributed to the altered levels of eicosanoids caused by the ingestion of n-3 fatty acids from fish oil. Because of the active participation of lung tissue in eicosanoid metabolism, this study was conducted to monitor the uptake and incorporation of the different n-3 fatty acids into lung phospholipid classes; to assess their effects on 20:4 n-6 levels; and to determine the effects of n-3 fatty acids on eicosanoid production and especially to ascertain if there is any selective decrease of prostaglandins synthesized by lungs.

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MATERIALS AND METHODS

Refined menhaden oil was obtained from Empire Menhaden Oil Co. Inc. (Empire, Louisiana). The sources of other chemicals were described in previous publications (11,16).

Animals

Male mice weighing 18 g were obtained from Charles River, Wilmington, Massachusetts. They were maintained at 20-22 C in cages in an approved animal facility. Each cage contained five mice. Animals had continuous access to food and water. A 12 hr dark and light cycle was maintained in the room.

Diets

The basic composition of the semisynthetic diet was dextrose (50%), casein (24%), AIN-mineral mix (6%), vitamin mix (2.2%), cerelose (5.48%), methionine (0.3%), BHT (0.01%), vitamin E (5 IU/g oil), zinc carbonate (0.01%) and safflower oil (2%). Safflower oil was used to provide essential fatty acids. The diets were adequate in all nutrients (17) and differed only in fat source. Regular mouse laboratory food was used as the control diet; it contained adequate amounts of saturated, monoene and polyene fatty acids. The semisynthetic diet was supplemented with hydrogenated coconut oil, 10% by weight (CO group) and used as the

reference for saturated fatty acid-enriched diet. The semisynthetic diet was supplemented with menhaden oil, 10% by weight, for the experimental (MO) group. The fatty acid compositions of dietary fats are summarized in Table 1. While menhaden oil-enriched diets contained high levels of n-3 fatty acids such as 20:5 and 22:6, the control and coconut oil-enriched diets were devoid of n-3 fatty acids. Animals received fresh diet every day and uneaten food was discarded. Autoxidation of dietary fats as measured by malondialdehyde formation (18) was negligible during the experiments. Food consumption (6 g/mouse/day) and body weight gain (7–8 g/mouse in two wk) during the course of experiments were similar in all groups. Animals were maintained on the special diets for two wk.

Preparation of Lung Microsomes

Mice were killed using ether anesthesia. The lungs were removed immediately, washed in cold saline, weighed and homogenized in five vol of 0.25 M sucrose-1 mM EDTA-10 mM Tris buffer (pH 7.4) using a polytron. Microsomes were prepared from homogenized lungs as described elsewhere (11). The microsomal fraction was suspended in 0.05 M Tris-HCl buffer, (pH 7.4, 2 ml/g lung) and immediately used for lipid analysis and measurement of eicosanoid synthesis.

Lipid Analysis

Microsomal lipids were extracted by the method of Bligh and Dyer (19). Phospholipid classes were separated by thin layer chromatography (TLC) on silica gel 60G (E. Merck, Darmstadt, West Germany) plates with a solvent system of chloroform/methanol/acetic acid/water (100:50:16:7, v/v/v/v) (20). Lipid classes were eluted from silica gel with chloroform/methanol (2:1, v/v) and saponified using 0.5 M KOH in methanol. Fatty acids were methylated using 14% BF_3 in methanol. Methyl esters were separated and identified by gas liquid chromatography (5880A, Hewlett-Packard, Avondale, Pennsylvania) using silar 10-C (Supelco Co., Bellefonte, Pennsylvania) column (11). Fatty acids were quantified using pentadecanoic acid as internal standard (21). Total phospholipids were estimated by the method of Stewart using dipalmitoyl phosphatidylcholine as reference standard (22). Cholesterol was measured by the method of Rudel and Morris (23).

TABLE 1

Fatty Acid Compositions of Dietary Fats

Fatty acid	wt %		
	Control diet	Coconut oil-enriched diet	Menhaden oil-enriched diet
12:0	2.8	41.3	—
14:0	1.7	16.5	8.2
16:0	21.5	9.9	15.3
16:1	2.8	—	—
18:0	12.1	12.3	4.7
18:1	35.3	3.4	14.7
18:2	21.1	16.6	17.7
18:3(n-6)	2.7	—	—
18:3(n-3)	—	—	2.4
18:4	—	—	3.3
22:1	—	—	0.2
20:4(n-6)	—	—	0.5
20:4(n-3)	—	—	1.0
20:5(n-3)	—	—	12.7
22:5(n-6)	—	—	1.1
22:5(n-3)	—	—	1.5
22:6(n-3)	—	—	4.9

Eicosanoid Synthesis from Arachidonic Acid

Lung microsomes (1 mg protein) in 0.3 ml of 0.05 M Tris-HCl buffer (pH 7.4) were incubated with 7.5 nmol of [^{14}C]arachidonic acid (0.01 μCi) at 37 C for 5 min and 15 min. Reactions were terminated by adding 3% formic acid to adjust the pH to 3.5. Eicosanoids were extracted three times in three vol of ethyl acetate and separated by TLC on silica gel 60G plates with a solvent system of chloroform/methanol/acetic acid/water (90:8:1:0.8, v/v/v/v) (24). The plates were developed twice in the same solvent system (11). The eicosanoids were visualized by spraying the plates with 10% phosphomolybdic acid in ethanol and heating the plates at 110 C for 10 min. The silica gel bands corresponding to authentic standards were scraped and counted in a liquid scintillation counter (Packard tricarb, model 3385, Packard, Rockford, Illinois).

The formation of eicosanoids from [^{14}C]-arachidonic acid and its separation on thin layer chromatograms also were visualized in autoradiograms after exposing the plates to X-ray films (Kodak x-omatic, Rochester, New York) as described elsewhere (11).

Miscellaneous Methods

Protein was estimated by the method of Lowry et al. (25) using bovine serum albumin as reference standard. The experimental data were analyzed with Student's t-test.

RESULTS AND DISCUSSION

Phospholipid and cholesterol content of lung microsomal lipids from mice consuming

coconut oil- and menhaden oil-enriched diets were comparable to those observed in control diet animals (Table 2).

Fatty Acid Modification of Lung Microsomes

Fatty acid compositions of the lung microsomes of mice on coconut oil-enriched diets were similar to those observed in animals maintained on normal lab chow diets (Tables 3-6). A significant increase in the n-3 fatty acids was observed in all major phospholipid classes of lung microsomes isolated from mice consuming menhaden oil. After two wk on menhaden oil-enriched diets, mice had significantly increased levels of 20:5 and 22:6 in phosphatidylcholine, phosphatidylethanolamine, phosphatidyl (inositol + serine) and neutral lipid fractions (Tables 3-6). This was accompanied by a three- to four-fold decrease in the arachidonic acid content of all the lipid classes of microsomes from mice on the menhaden oil. In addition, there was a significant decrease in 22:4 n-6 and 22:5 n-6 fatty acids in the phospholipid classes (Tables 3-5).

These studies indicated that the enrichment of n-3 fatty acids in lung microsomes of animals consuming menhaden oil occurred at the expense of arachidonic acid and other n-6 fatty acids. The decrease in arachidonic acid content in the menhaden oil-fed animals could not be attributed solely to the lack of conversion of 18:2 n-6 to 20:4 n-6 or to the low levels of 18:2 in precursor pools, since adequate amounts of this fatty acid were provided in the diet. Because of the close correlation between the decrease in arachidonic acid and other long chain n-6 fatty acids and an increase in n-3 fatty acids, it appears that n-3 fatty acids compete with n-6 fatty acids at the acylation level. Preferential uptake and desaturation of n-3 compared to n-6 fatty acids have been observed in tissues like heart, platelets, skeletal muscle and liver membranes (26-28).

The extensive modification of microsomal lipid fatty acids following the consumption of menhaden oil-containing diets was restricted to

TABLE 2

Phospholipid and Cholesterol Contents of *Mouse Lung Microsomes*

	n moles/mg protein		
	Control diet	Coconut oil-enriched diet	Menhaden oil-enriched diet
Phospholipid	426 ± 29	457 ± 16	445 ± 37
Cholesterol	47 ± 7	46 ± 7	52 ± 4

Mean ± SD, n = 4.

TABLE 3

Effect of Dietary Coconut Oil and Menhaden Oil on Fatty Acid Composition of Lung Microsomal Phosphatidylcholine

	n moles/mg protein			
Fatty acid	Control	Coconut oil-enriched diet	Menhaden oil-enriched diet	P
12:0	—	—	—	
14:0	1.7 ± 0.4	7.9 ± 2.6	4.7 ± 1.3	
16:0	45.7 ± 7.8	59.9 ± 8.6	51.7 ± 7.9	
16:1	7.9 ± 1.6	10.8 ± 1.7	9.0 ± 1.1	
18:0	7.2 ± 1.0	12.6 ± 3.7	8.5 ± 2.8	
18:1	12.7 ± 2.4	14.2 ± 1.9	11.1 ± 1.5	
18:2 (n-6)	5.1 ± 0.9	5.6 ± 1.0	4.0 ± 0.8	
18:3 (n-3)	0.5 ± 0.1	0.4 ± 0.3	0.3 ± 0.2	
18:4	—	0.6 ± 0.3	0.6 ± 0.2	
22:1	0.12 ± 0.1	0.8 ± 0.4	0.3 ± 0.1	
20:4 (n-6)	8.2 ± 0.5	7.9 ± 1.9	2.4 ± 0.8***[a]	<0.001
20:5 (n-3)	—	0.3 ± 0.09	1.5 ± 0.3***	<0.001
22:4 (n-6)	0.4 ± 0.04	1.2 ± 0.3	0.2 ± 0.03**	<0.01
22:5 (n-6)	0.24 ± 0.1	0.8 ± 0.3	0.1 ± 0.02**	<0.01
22:5 (n-3)	0.8 ± 0.4	—	1.3 ± 0.6	
22:6 (n-3)	0.34 ± 0.06	1.3 ± 0.4	3.0 ± 0.2***	<0.001
Saturates	62%	65%	66%	
Monounsaturates	24%	21%	21%	
Polyunsaturates	14%	14%	13%	
n-6/n-3 ratio	9.1	7.75	1.15	

Mean ± SD of five experiments.

[a]Asterisks denote significant differences compared to control diets (see "P" column).

TABLE 4

Effect of Dietary Coconut Oil and Menhaden Oil on Fatty Acid Composition of Lung Microsomal Phosphatidylethanolamine

Fatty acid	n moles/mg protein			P
	Control	Coconut oil-enriched diet	Menhaden oil-enriched diet	
14:0	0.6 ± 0.2	1.1 ± 0.6	1.1 ± 0.3	
16:0	13.4 ± 2.5	9.2 ± 1.1	9.9 ± 1.3	
16:1	1.9 ± 0.4	3.8 ± 0.6	3.4 ± 0.6	
18:0	9.2 ± 1.2	7.2 ± 1.0	6.5 ± 0.5	
18:1	9.6 ± 1.9	5.9 ± 0.7	5.5 ± 0.9	
18:2 (n-6)	3.4 ± 0.7	1.8 ± 0.3	1.6 ± 0.3	
18:3 (n-3)	0.9 ± 0.3	0.4 ± 0.3	0.3 ± 0.1	
18:4	—	0.4 ± 0.3	0.3 ± 0.2	
22:1	—	0.6 ± 0.3	0.4 ± 0.2	
20:4 (n-6)	12.4 ± 1.9	9.5 ± 2.0	3.1 ± 0.3***[a]	<0.001
20:5 (n-3)	0.5 ± 0.4	0.5 ± 0.3	2.3 ± 0.3***	<0.001
22:4 (n-6)	3.9 ± 0.6	3.5 ± 0.9	0.7 ± 0.2***	<0.001
22:5 (n-6)	1.2 ± 0.3	1.9 ± 0.7	0.5 ± 0.2**	<0.01
22:5 (n-3)	1.7 ± 0.8	0.7 ± 0.4	4.3 ± 0.4***	<0.001
22:6 (n-3)	3.4 ± 0.7	2.4 ± 0.7	6.5 ± 0.9***	<0.001
Saturates	35%	36%	38%	
Monounsaturates	18%	21%	20%	
Polyunsaturates	47%	43%	42%	
n-6/n-3 ratio	3.2	4.21	0.44	

Mean ± SD of five experiments.

[a]Asterisks denote significant differences compared to control diets (see "P" column).

TABLE 5

Effect of Dietary Coconut Oil and Menhaden Oil on Fatty Acid Composition of Lung Microsomal (Serine + Inositol) Phospholipids

Fatty acid	n moles/mg protein			P
	Control	Coconut oil-enriched diet	Menhaden oil-enriched diet	
14:0	0.5 ± 0.3	1.2 ± 0.9	1.7 ± 1.1	
16:0	4.3 ± 1.4	3.0 ± 0.6	3.4 ± 1.2	
16:1	0.7 ± 0.2	0.5 ± 0.1	0.5 ± 0.2	
18:0	12.6 ± 1.8	14.8 ± 5.2	14.4 ± 3.1	
18:1	7.2 ± 1.9	4.4 ± 1.8	4.6 ± 1.2	
18:2 (n-6)	1.9 ± 0.6	0.6 ± 0.3	0.6 ± 0.3	
18:3 (n-3)	0.5 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	
18:4	—	0.1 ± 0.05	0.1 ± 0.06	
22:1	0.3 ± 0.06	0.7 ± 0.3	0.6 ± 0.02	
20:4 (n-6)	5.7 ± 0.9	5.3 ± 2.7	2.5 ± 1.1**[a]	<0.01
20:5 (n-3)	—	0.3 ± 0.1	0.9 ± 0.2***	<0.001
22:4 (n-6)	1.7 ± 0.4	1.8 ± 0.5	0.6 ± 0.2***	<0.001
22:5 (n-6)	0.5 ± 0.1	1.0 ± 0.3	0.3 ± 0.1**	<0.01
22:5 (n-3)	0.4 ± 0.3	—	2.6 ± 0.3***	<0.001
22:6 (n-3)	0.9 ± 0.1	1.1 ± 0.4	2.7 ± 0.4***	<0.001
Saturates	44%	55%	55%	
Monounsaturates	20%	16%	16%	
Polyunsaturates	36%	29%	29%	
n-6/n-3 ratio	5.3	5.7	0.63	

Mean ± SD of five experiments.

[a]Asterisks denote significant differences compared to control diets (see "P" column).

TABLE 6

Effect of Dietary Coconut Oil and Menhaden Oil on Fatty Acid Composition of Lung Microsomal Neutral Lipids

Fatty acid	n moles/mg protein			P
	Control	Coconut oil-enriched diet	Menhaden oil-enriched diet	
12:0	1.4 ± 0.1	10.0 ± 2.1	1.2 ± 0.2	
14:0	2.9 ± 0.7	17.3 ± 2.9	11.8 ± 0.6	
16:0	49.6 ± 5.1	98.1 ± 14.3	85.7 ± 7.2	
16:1	13.2 ± 3.5	32.4 ± 8.0	27.7 ± 1.6	
18:0	11.9 ± 0.6	21.9 ± 2.6	17.5 ± 1.5	
18:1	61.6 ± 9.6	83.6 ± 13.9	62.6 ± 4.4	
18:2 (n-6)	15.6 ± 1.8	18.7 ± 3.2	20.2 ± 2.1	
18:3 (n-3)	1.8 ± 0.4	1.3 ± 0.5	3.0 ± 0.9	
18:4	—	0.7 ± 0.5	1.6 ± 0.6	
22:1	—	1.0 ± 0.5	0.8 ± 0.6	
20:4 (n-6)	4.9 ± 0.5	5.7 ± 1.3	2.8 ± 1.2**[a]	<0.01
20:5 (n-3)	—	0.9 ± 0.2	3.8 ± 1.1**	<0.01
22:4 (n-6)	0.9 ± 0.5	1.7 ± 1.5	—*	<0.05
22:5 (n-6)	0.6 ± 0.4	2.1 ± 1.1	3.5 ± 2.5	
22:5 (n-3)	0.3 ± 0.2	1.1 ± 1.0	5.1 ± 2.1**	<0.01
22:6 (n-3)	0.5 ± 0.1	2.5 ± 2.2	5.9 ± 2.9*	<0.05
Saturates	49%	49%	48%	
Monounsaturates	37%	39%	36%	
Polyunsaturates	14%	12%	16%	
n-6/n-3 ratio	7.5	5	1.48	

Mean ± SD of five experiments.

[a]Asterisks denote significant differences compared to control diets (see "P" column).

polyunsaturated fatty acids with chain lengths greater than 20 carbon atoms. However, there was no selectivity in the incorporation of n-3 fatty acids into lung lipids. In addition, the ratio of polyunsaturated fatty acids to saturated fatty acids remained constant in animals on the control, coconut oil and menhaden oil diets. However, the ratio of n-6 to n-3 fatty acids decreased drastically in animals consuming menhaden oil.

Effect of Fatty Acid Modification on Eicosanoid Formation

Microsomes from mice lung synthesize a range of prostaglandins and hydroxy fatty acids from exogenous arachidonic acid (Fig. 1). Indomethacin inhibited the synthesis of cyclooxygenase products (zones 3, 4 and 5 in column B, Fig. 1). Microsomes heated at 100 C for 5 min failed to synthesize eicosanoids from arachidonic acid (column C, Fig. 1), indicating the enzymatic nature of the reaction.

The syntheses of prostaglandin E_2, prostaglandin $F_{2\alpha}$ and thromboxane B_2 from exogenous arachidonic acid by lung microsomes of animals on control diets and coconut oil-enriched diets were comparable (Fig. 2). However, the ability of n-3 fatty acid-enriched lung microsomes from animals consuming menhaden oil to synthesize prostaglandin E_2 and prostaglandin $F_{2\alpha}$ from arachidonic acid was decreased significantly compared to those in mice maintained on lab chow or coconut oil-enriched diets (Fig. 2). Though thromboxane was produced in lower amounts by lung microsomes from the menhaden oil group, it was not statistically significant compared to that in control or coconut oil groups. Similarly, hydroxy fatty acid formation was comparable in all groups (Fig. 2).

These results are in general agreement with those reported by Hansen et al., who showed a drastic reduction in the prostaglandin E_2 formation from chopped lungs obtained following administration of ethyl linolenate (n-3) to rats (3). Marshall and Johnston observed that dietary supplementation with α-linolenic acid (18:3 n-3) significantly decreased the formation of prostaglandin $F_{2\alpha}$ in liver and prostaglandin E_2 in peritoneal macrophages from rats (29,30). Meydani et al. observed that supplementation of diets with safflower oil (n-6 fatty acid) increased the formation of prostaglandin $F_{2\alpha}$ (8-fold), prostaglandin E_2 (13-fold) and prostaglandin E_1 (11-fold) in rat lung homogenates (31). These studies indicated that while diet supplementation with n-3 fatty acids decreases the formation of prostaglandins of two series, n-6 fatty acid supplementation

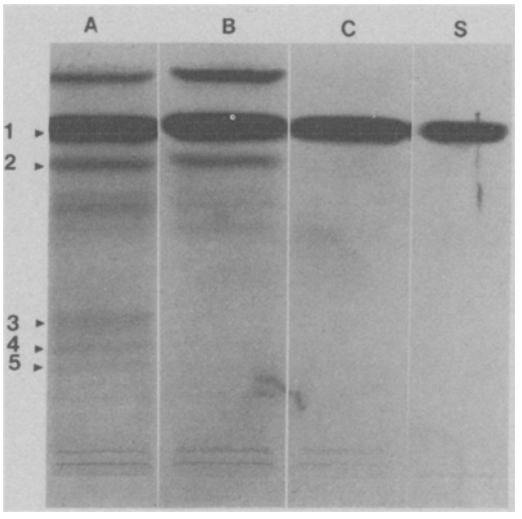

FIG. 1. Autoradiogram of eicosanoid formation by mouse lung microsomes incubated with [^{14}C]arachidonic acid. Column A: lung microsomes (1 mg protein) from mice on control diets were incubated with [^{14}C]arachidonic acid (0.01 μCi/7.5 n moles) at 37 C for 15 min. After the medium was acidified with 3% formic acid, eicosanoids were extracted into ethyl acetate and separated on thin layer chromatogram as described in Materials and Methods. The thin layer plates were exposed to X-ray films for three days before developing the films. The individual prostaglandins were identified by comparing their R_f values with authentic standards. Zone 1, arachidonic acid; Zone 2, hydroxy fatty acid; Zone 3, prostaglandin E_2; Zone 4, thromboxane B_2 and Zone 5, prostaglandin $F_{2\alpha}$. Column B: experimental conditions are as described for the microsomes in column A except 1 μM of indomethacin was included in the reaction mixture. Column C: experimental conditions are as described for the microsomes in column A except microsomes inactivated in boiling water bath for 5 min were used. Column S: [^{14}C]standard arachidonic acid.

enhances their production. Hence the availability of n-6 fatty acids in tissue seems to regulate synthesis of prostaglandins. In support of this hypothesis, it has been demonstrated that n-3 fatty acids in rat diets decreases 20:4 n-6 levels in the liver, spleen and macrophages (28-30). A decrease in the level of 20:4 n-6 subsequently decreases the formation of prostaglandins (29,30). When arachidonic acid was supplied exogenously, no differences were observed in prostaglandin production of n-3 and n-6 fatty acid-enriched tissues (29,30). However, Marshall and Johnston observed that the addition of free eicosapentaenoic acid (20: n-3) to n-6 fatty acid-enriched liver homogenates significantly decreased production of the two series prostaglandins (29), indicating that in addition to the availability of arachidonic acid, competition of eicosapentaenoic acid (n-3 fatty acid) with arachidonic acid for cyclooxygenase could influence prostaglandin production.

Our current studies indicate that, unlike liver, the ability of n-3 fatty acid-enriched lung microsomes to synthesize prostaglandin E_2 and $F_{2\alpha}$ from exogenous arachidonic acid is still lower than that observed with lung microsomes low in n-3 fatty acids. This and previous studies with rats and guinea pigs (11) indicate that in lungs not only the availability of arachidonic acid, but also the presence of n-3 fatty acids such as eicosapentaenoic and docosahexaenoic acid, alters the capacity of lungs to synthesize two series prostaglandins.

The reason for the decreased cyclooxgenase activity caused by dietary menhaden oil is not clear. Meydani et al. (31) and Mathias and Dupont (32) are of the opinion that the ratio of polyunsaturated to saturated fatty acids may regulate prostaglandin formation in rat lungs. Our studies with mice and previous observations on rats and guinea pigs (11) demonstrate that n-3 fatty acid-enriched lung microsomes produce lower amounts of prostaglandin E_2 and prostaglandin $F_{2\alpha}$, not because of the altered polyunsaturated to saturated fatty acid ratio, but because of a decreased n-6/n-3 fatty acid ratio. This indicates that it is the type of polyunsaturated fatty acid which can influence prostaglandin production in lungs. The n-3 polyunsaturated fatty acids (eicosapentaenoic and docosahexaenoic) may alter membrane fluidity and affect cyclooxygenase activity (33). In addition, these act as potent competitive inhibitors of cyclooxygenase enzyme with K_i of 2.5 μM and 1.7 μM, respectively (34). The enrichment of such fatty acids in membrane phospholipids may exert inhibitory effects on cyclooxygenase by competing with arachidonic acid for eicosanoid formation (35,36).

Prostaglandins are known to play a key role in pulmonary functions, including dilation and constriction of bronchial vessels, which could have direct impact on asthma (1,37). The ability of lungs to produce prostaglandins must be carefully regulated to maintain the appropriate balance of these physiologically active compounds. Environmental factors and physiologically active substances influence prostaglandin production by lungs (38,39). As shown in our current studies, dietary fats can significantly influence the cyclooxygenase activity of lungs. Hence nutritional factors could have a significant impact on pulmonary functions mediated by prostaglandins and leukotrienes. Advocacy for increased fish oil ingestion to ameliorate thrombosis must be

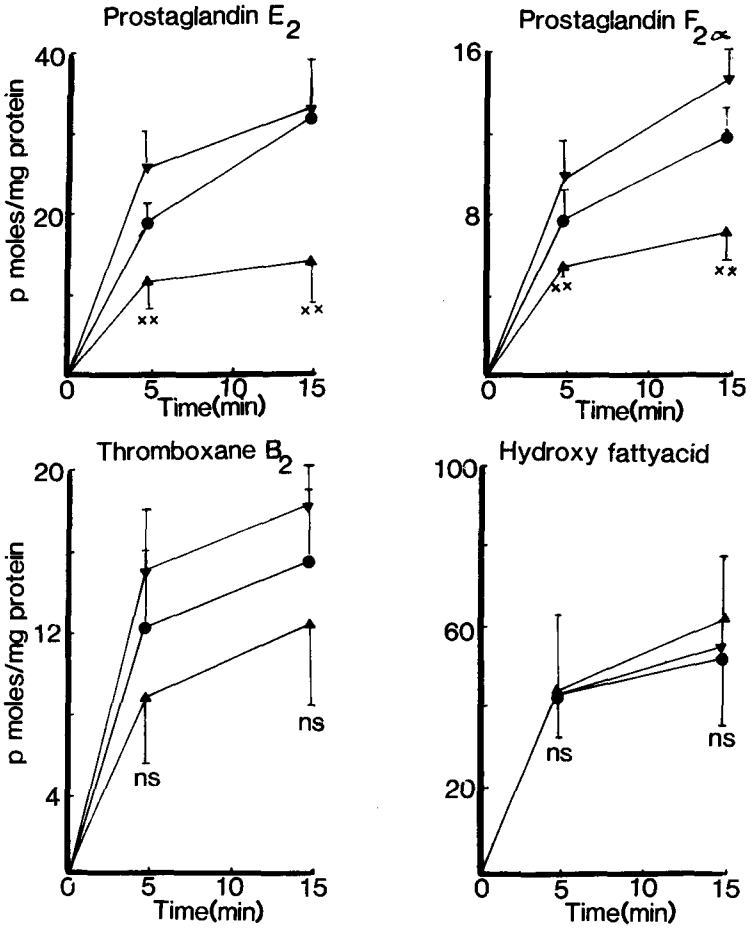

FIG. 2. Eicosanoid formation from arachidonic acid by lung microsomes isolated from mice on control (▼—▼), coconut oil-enriched diet (● — ●) and menhaden oil-enriched diet (▲—▲). Lung microsomes (1 mg protein) were incubated with [^{14}C]arachidonic acid (0.01 μCi/7.5 n moles) for the time intervals indicated. Labeled eicosanoids formed were extracted in ethyl acetate and quantitated as described in Materials and Methods. Values are mean ± SD of five experiments. xx = $p < 0.01$, ns = not significant.

tempered by caution concerning the potential impact on prostaglandin production in other vital organs such as lungs, kidneys and liver.

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Suppression by Dexamethasone of Isoproterenol-Mediated Changes in Fatty Acyl-CoA Desaturase Activity of *Tetrahymena* Microsomes

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ABSTRACT

Preincubation of *Tetrahymena pyriformis* cells with dexamethasone inhibited the microsomal fatty acyl-CoA desaturase activities of isoproterenol-induced modulation; that is, an increase in Δ^9-desaturase activity accompanied by a decrease in Δ^{12}-desaturase activity. Although isoproterenol caused an increase in Δ^9-terminal component activity with decreased Δ^{12}-terminal component activity, dexamethasone reduced these isoproterenol-mediated activity changes. In cells treated with dexamethasone prior to isoproterenol administration, stimulation of cyclic AMP accumulation by isoproterenol was inhibited. These results suggest that dexamethasone may repress isoproterenol modulation of the activity of terminal components (cyanide-sensitive factor) in the fatty acyl-CoA desaturase system by reducing the cyclic AMP level.
Lipids 20:850–853, 1985.

INTRODUCTION

Differences have been shown between the effects of glucocorticoid excess and treatment with glucocorticoids in deficiency. When adrenalectomized animals are treated with glucocorticoids, there is an enhancement of the responsiveness to catecholamines in a variety of cells, such as lipolytic actions (1) and β-adrenergic responsiveness (2,3). Ingle (4) proposed the term "permissive" for the role of glucocorticoids in facilitating cell responses to many hormones. An excessive administration of glucocorticoids induces insulin resistance and glucose intolerance in man and experimental animals (1,5,6). However, little is known about the effects of glucocorticoid excess on cyclic AMP-mediated processes in cells.

We recently have obtained evidence that a unicellular eukaryote, *Tetrahymena pyriformis*, is highly responsive to β-adrenergic agonists and glucocorticoids, and that its microsomal fatty acyl-CoA desaturation system is modulated strongly by these hormones (7). In the present study, we investigated the effects of a glucocorticoid, dexamethasone, on isoproterenol-induced modulation of the fatty acyl-CoA desaturase system in *Tetrahymena* microsomes.

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MATERIALS AND METHODS

Materials

The following chemicals were obtained from commercial sources: [1-^{14}C]stearoyl-CoA (59.6 mCi/mmol) and [1-^{14}C]oleoyl-CoA (59.7 mCi/mmol) (New England Nuclear, Boston, Massachusetts); cyclic AMP radioimmunoassay kit (Yamasa Shoyu Co. Ltd., Tokyo); stearoyl-CoA and oleoyl-CoA (P-L Biochemicals Inc., Milwaukee, Wisconsin), and NADH, (±)-isoproterenol, theophylline and dexamethasone (Sigma Chemical Co., St. Louis, Missouri). Other chemicals were of the highest purity available from commercial sources.

Cell Growth and Isolation of Microsomes

A thermotolerant strain NT-1 of *Tetrahymena pyriformis* was grown with continuous shaking (90 cycles/min) at 39 C in 500 ml Erlenmeyer flasks containing 200 ml of an enriched proteose-peptone medium (2% proteose-peptone, 0.2% yeast extract and 90 μM EDTA-Fe^{3+} complex) without D-glucose (8). When the density of cells reached 4.0×10^5 cells/ml, a midexponential phase, 400 μM theophylline plus 100 μM isoproterenol was added to the medium, and the culture was incubated for the indicated periods at 39 C. Control cells received only 400 μM theophylline. In experiments with dexamethasone, 10 μM dexamethasone was administered 30 min prior to the addition of isoproterenol. At the required time, cells were

homogenized in 5 vol of phosphate buffer (0.2 M K_2HPO_4/0.2 M KH_2PO_4, 3mM EDTA and 0.1 M NaCl, pH 7.4) with a hand glass homogenizer (A. H. Thomas Co., USA), and microsomal fractions were prepared from homogenates as previously described (9). The microsomes obtained were washed once by suspension in 50–100 ml of fresh 0.1 M potassium phosphate buffer (pH 7.4). Washed microsomes were frozen immediately at −90 C before use. Protein was determined according to the method of Lowry et al. (10).

Assay of Fatty Acyl-CoA Desaturases

For assays of Δ^9- and Δ^{12}-desaturase activities, typical reaction mixtures contained 20 nmol of [1-^{14}C]stearoyl-CoA (1 Ci/mol) or [1-^{14}C]oleoyl-CoA (1 Ci/mol) as substrate, 50 nmol of NADH as an electron donor, suitable amounts of microsomes (0.10 mg for the stearoyl-CoA desaturase assay and 0.40 mg for the oleoyl-CoA desaturase assay) and 0.1 M potassium phosphate buffer (pH 7.2) to a final volume of 0.5 ml. Each sample was preincubated for one min at 39 C prior to the addition of microsomes to initiate the reaction. After incubation for three min at 39 C, the reaction was stopped by the addition of 0.5 ml of 10% KOH in methanol. Analysis of reaction products was performed as described previously (7,11–13). Labeled fatty acid methyl esters separated by a JEOL Model JGC-1100 gas chromatograph were trapped by insertion of glass tubes (5 × 150 mm) with open ends and eluted from the tubes with toluene into scintillation vials. Radioactivity was determined with a Beckman scintillation counter.

Determination of Reoxidation Rate of Cytochrome b_5

The terminal component of the desaturase system was assayed spectrophotometrically, determining the stimulation of cytochrome b_5 oxidation by the addition of stearoyl-CoA (or oleoyl-CoA). The sample cuvette obtained contained microsomal protein with 0.2 nmol of cytochrome b_5, 0.1 M Tris-HCl (pH 7.2), and 200 nmol of fresh Na_2S in a final volume of 3.0 ml. The rate of fatty acyl-CoA-stimulated reoxidation of cytochrome b_5 reduced by 2 nmol of NADH was calculated using 216 $mM^{-1} \cdot cm^{-1}$ as the extinction difference of the cytochrome between 425 and 410 nm as described previously (14,15).

Cyclic AMP Measurement

Cyclic AMP content was measured by the method of Steiner et al. (16) with a cyclic AMP radioimmunoassay kit. At the times indicated, cells were harvested by centrifuging cell medium containing over 2×10^7 cells/medium. The washed cells were resuspended in 5 ml of 6% trichloroacetic acid cooled at 0–2 C. The suspension then was sonicated for two min and left in an ice bath for one hr. The samples were prepared as described previously (7). The assay mixture contained 100 μl of succinylated sample, 50 mM imidazole buffer (pH 6.5), 100 μl of anticyclic AMP antibody from a rabbit and 16 pCi of a tyrosine methyl ester derivative of succinyl cyclic [^{125}I]AMP and was kept cold for 18 hr. Its radioactivity was determined with a Packard Model 5650 Autogamma counter.

RESULTS AND DISCUSSION

A unicellular eukaryote, *Tetrahymena pyriformis*, has been shown to be capable of synthesizing catecholamines (17) and of responding to them (18) and glucocorticoids (19). It generally has been shown that glucocorticoids alter the responsiveness of cells to β-adrenergic agonists (1–3). Therefore, investigation of the influence of glucocorticoids on the β-adrenergic responsiveness of fatty acyl-CoA desaturase system seems worthwhile.

Table 1 indicates the effects of isoproterenol and dexamethasone on the fatty acyl-CoA desaturase activity in *Tetrahymena* microsomes. Although dexamethasone and a β-adrenergic agonist, isoproterenol, produced increases in stearoyl-CoA desaturase activity concomitant with decreases in oleoyl-CoA desaturase activity, this isoproterenol-induced modification was dose-dependently inhibited by preincubation with dexamethasone prior to isoproterenol administration.

The microsomal fatty acyl-CoA desaturase system consists of multicomponent membrane-bound enzymes containing at least cytochrome b_5 reductase, cytochrome b_5 and the terminal component (cyanide-sensitive factor). Isoproterenol and dexamethasone had no significant effects on the activities of NAD(P)H-cytochrome *c* and NADH-ferricyanide reductases and the content of cytochrome b_5 (data not shown). Nevertheless, dexamethasone inhibited isoproterenol-induced modification of the terminal component activities, namely, a two-fold increase in Δ^9-terminal component activity and a two-fold decrease in Δ^{12}-terminal component activity over the first two hr after the addition of isoproterenol (Fig. 1). These findings suggest that the terminal component, which is most sensitive to hormones among the constituents of the fatty acyl-CoA desaturase system, may

TABLE 1

Changes by Various Drugs on *Tetrahymena* Microsomal Fatty Acyl-CoA Desaturase Activities

Conditions	Activity after drug additions and incubation			
	0 hr (n = 3)	1 hr (n = 3)	2 hr (n = 3)	3 hr (n = 2)
		(nmol/min/mg protein)		
Δ^9-desaturase activity				
Theophylline alone (A)	4.31 ± 0.44	5.06 ± 0.43	5.31 ± 0.42	6.28
A + 10 μM dexamethasone		7.01 ± 0.46*	8.75 ± 0.52*	7.70
A + 100 μM isoproterenol (B)		8.02 ± 0.57*	9.69 ± 0.61*	9.13
A + 400 μM isoproterenol		7.68 ± 1.20	9.41 ± 1.64*	8.88
1 μM dexamethasone prior to B		7.12 ± 1.81	8.08 ± 1.55	7.86
10 μM dexamethasone prior to B		5.25 ± 0.69	6.03 ± 0.46	6.82
Δ^{12}-desaturase activity				
A	0.94 ± 0.06	0.90 ± 0.04	0.83 ± 0.07	0.75
A + 10 μM dexamethasone		0.67 ± 0.07*	0.43 ± 0.05*	0.60
A + B		0.77 ± 0.09*	0.50 ± 0.05	0.69
A + 400 μM isoproterenol		0.72 ± 0.08*	0.55 ± 0.10*	0.67
1 μM dexamethasone prior to B		0.80 ± 0.10	0.63 ± 0.08	0.68
10 μM dexamethasone prior to B		0.84 ± 0.07	0.76 ± 0.08	0.72

Details for enzyme assays are described in Materials and Methods. Theophylline (400 μM) and/or various drugs were added to a culture when the cell density reached a midexponential phase. For examination of repression by dexamethasone of isoproterenol-induced modification of fatty acyl-CoA desaturase activities, 1 and 10 μM dexamethasone were added 30 min prior to the addition of isoproterenol. Values are the average ± S.D. n, Number of experiments performed. *, Values differ significantly from values (A) at the corresponding periods, $p < 0.01$.

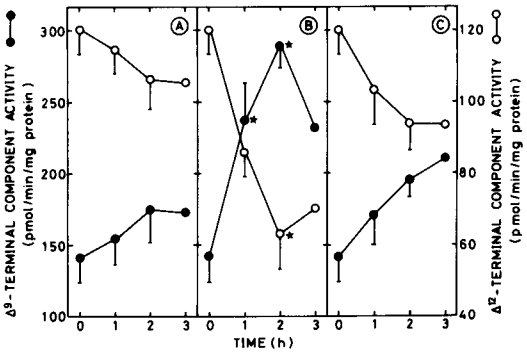

FIG. 1. Dexamethasone inhibition of isoproterenol-induced modulation of Δ^9- and Δ^{12}-terminal component activities in *Tetrahymena* microsomes. Details for the enzyme assays are described in the text. Figure shows the time course of the component activities after treatment with isoproterenol and/or theophylline. Each point is the average (±S.D.) of 2 (or 3) separate experiments done in duplicate. A, only 400 μM theophylline was added (control cells); B, 100 μM isoproterenol and 400 μM theophylline were added; C, 10 μM dexamethasone was added 30 min prior to addition of isoproterenol and theophylline. ●—●, Δ^9-terminal component activity; ○—○, Δ^{12}-terminal component activity. *, Values differ significantly from control values at the corresponding periods in Fig. 1A, $p < 0.01$.

play a principle role in the regulation of overall desaturation activity.

The results illustrated in Table 2 show an isoproterenol-induced cyclic AMP accumulation and its inhibition by dexamethasone. This inhibition of cyclic AMP production may result mainly from the suppression by dexamethasone of isoproterenol-induced stimulation of adenylate cyclase activity. This possibility also is supported by evidence that similar changes have been observed in experiments with epinephrine substituted for isoproterenol (20). These observations are consistent with a recent report that an increased cyclic AMP production induced by vasoactive intestinal peptide (VIP) was concentration-dependently inhibited by dexamethasone (21). The data obtained in this investigation indicate that dexamethasone alters the β-adrenergic responsiveness of *Tetrahymena* cells. It is known that glucocorticoids can penetrate the cell (19) and bind to plasma membrane (22,23). In addition, Lai et al. (3) have demonstrated that glucocorticoids change the number of β-adrenergic receptors on the plasma membrane of 3T3-L1 preadipocytes and adipocytes and the sensitivity of adenylate cyclase to β-adrenergic agonists. Based on these findings, one might speculate that the effects of dexamethasone reflect the action on a possible specific β-adrenergic receptor on plasma membrane of *Tetrahymena* under the present

TABLE 2

Dexamethasone Inhibition of Isoproterenol-Induced Cyclic AMP Accumulation in *Tetrahymena*

Conditions	Time after drug additions				
	0 hr (n = 3)	0.5 hr (n = 2)	1 hr (n = 3)	2 hr (n = 3)	3 hr (n = 2)
			(× 10^5 cells/ml)		
Cell density	35 ± 7	40	46 ± 44	57 ± 5	63
Cyclic AMP content			(pmol/10^6 cells)		
Theophylline alone (A)	1.92 ± 0.71	2.27	3.08 ± 0.44	3.41 ± 0.39	2.44
A + 10 μM dexamethasone		4.82	6.02 ± 0.55*	6.65 ± 0.41*	5.36
A + 100 μM isoproterenol (B)		6.29	7.08 ± 0.47*	8.11 ± 0.32*	5.91
A + 400 μM isoproterenol		6.78	7.22 ± 0.68*	7.87 ± 0.56*	6.08
1 μM dexamethasone prior to B		5.40	6.81 ± 1.10*	7.05 ± 0.66*	4.28
10 μM dexamethasone prior to B		3.82	4.03 ± 0.97	4.92 ± 0.82	3.47

Details for enzyme assays are described in Materials and Methods. 400 μM theophylline and/or various drugs were added to a culture when the cell density reached a midexponential phase. One and 10 μM dexamethasone were added 30 min prior to the addition of 100 and 400 μM isoproterenol. Values are the average ± S.D. n, Number of experiments performed. *, Values differ significantly from control values (A) at the corresponding periods, $p < 0.01$.

experimental conditions. Sequentially, the observations from this study could allow us to conclude that dexamethasone may repress the modulation of activities of the terminal component, which plays an important role in the fatty acyl-CoA desaturase system, by acting through isoproterenol-induced activation of cyclic AMP-mediated processes. Such effects of dexamethasone appear to be different from the so-called permissive effect of glucocorticoids on cyclic AMP-mediated processes in their deficiency (e.g., increases in β-adrenergic responsiveness [1-3] and decreases in sensitivity to insulin [24,25]). On the other hand, dexamethasone produced an increase in stearoyl-CoA desaturase activity with a decrease in oleoyl-CoA desaturase activity via its elevation of intracellular cyclic AMP content (Tables 1 and 2). These results suggest that, although dexamethasone alone stimulated intracellular cyclic AMP accumulation, dexamethasone would have some biphasic (or counter) action in cyclic AMP accumulation following the addition of β-adrenergic agonists. To verify these suggestions, experiments with cyclic AMP analogs, substituted for β-adrenergic agonists, are under investigation. However, to achieve a more comprehensive understanding of the control mechanism for the observed repression by dexamethasone of isoproterenol-induced modulation of the fatty acyl-CoA desaturase system, further studies are required.

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Fluorescent Pigments by Covalent Binding of Lipid Peroxidation By-Products to Protein and Amino Acids

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ABSTRACT

The fluorescent products formed on reaction of 12-oxo-*cis*-9-octadecenoic acid (12-keto-oleic acid) with about 20 different amino acids, polylysine and bovine serum albumin (BSA) were studied. Besides glycine, only the basic amino acids histidine, lysine and arginine gave products with strong fluorescence. N-Acetylation of amino acids greatly reduced the fluorescence of their reaction products. The formation of fluorescent products was inhibited strongly by SH-amino acids such as N-acetyl-cysteine and glutathione. Polyacrylamide gel electrophoresis showed that BSA treated with 12-keto-oleic acid was more acidic than untreated or ricinoleic acid-treated BSA, indicating that basic amino acid residues in BSA were modified by reaction with the keto fatty acid. None of the structural analogs of 12-keto-oleic acid tested—12-oxo-*trans*-10-octadecenoic acid, 12-oxo-octadecanoic acid, 12-hydroxy-*cis*-9-octadecenoic acid (ricinoleic acid), *cis*-9-octadecenoic acid (oleic acid) and linoleic acid—reacted with glycine to give a fluorescent product. The fluorescent products formed on reaction of 12-keto-oleic acid methyl ester with benzyl amine and glycine methyl ester were shown to be 8-(N-substituted-4,5-dihydro-4-oxo-5-hexyl-5-hydroxy-2-pyrrolyl) octanoic acid methyl esters. The fluorescence properties of these compounds were attributed to the chromophobic system NC=CC=O which contains 6π electrons. This investigation contributes to insight of the mechanism of formation of fluorescent pigments, probably by a similar reaction of other compounds of the β,γ-unsaturated carbonyl type.

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INTRODUCTION

Lipofuscin and ceroid pigments are known to be associated with aging (1–6) or E hypovitaminosis (7–11) in animal tissues. These fluorescent substances generally are considered to consist of peroxidized lipid-protein complexes (12–14). Their characters and structures have been investigated by in vitro studies on the reactions of proteins with peroxidized lipids, but still are obscure. One of these compounds is thought to be a conjugated Schiff base product formed by the reaction of protein amino groups with α,β-unsaturated carbonyl groups of malondialdehyde, a decomposition product of peroxidized lipids (15).

Oral 12-oxo-*cis*-9-octadecenoic acid (12-keto-oleic acid: 12-KOA) is reported to enhance the incidence of encephalomalacia in chicks receiving a vitamin E-deficient diet (16,17). Moreover, accumulation of fluorescent pigments was observed in endotherial cells of cerebellar and cerebral vessels in encephalomalacic chicks (10). 12-KOA also was reported to have toxic effects, such as inactivation of enzymes (18) and acceleration of the formation of lipid peroxides (19.20) and fluorescent substances (21).

In the present study, we investigated the formation mechanism and the characterization of fluorescent pigments by covalent binding of 12-KOA, used as a model of secondary products of lipid peroxidation with β,γ-unsaturated carbonyl structure (22–24), to protein and amino acids.

MATERIALS AND METHODS

Materials

12-KOA and 12-oxo-*trans*-10-octadecenoic acid (conjugated 12-keto acid: 12-KCA) were prepared by the method of Nichols and Schipper (25). The methyl ester of 12-KOA was prepared by addition of diazomethane and purified by silicic acid column chromatography. 12-oxo-octadecanoic acid (12-keto stearic acid: 12-KSA) was prepared by hydrogenation of 12-KOA under a stream of hydrogen gas with 5% Pd on carbon as catalyst. BSA (fatty acid free), poly-L-lysine (M.W. 55000), amino acids and N-acetylated amino acids were purchased from Sigma Chemical Co. (St. Louis, Missouri). Benzylamine was obtained from Nakarai Chemical Co. (Tokyo, Japan).

Reaction of 12-KOA with BSA, Polylysine and Amino Acids

A solution of 12-KOA in chloroform in a test

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tube was evaporated under nitrogen. Then 1 ml of BSA in 0.1 M phosphate buffer, pH 7.4, was added to the dry lipid and shaken on a vortex mixer for one min. The final concentration of 12-KOA was 1 mM and that of BSA was 3 mg/ml. A ethanol solution of 12-KOA (0.4 ml) was added to 40 ml of polylysine in 0.1 M phosphate buffer, pH 7.4. The final concentrations of 12-KOA and polylysine were 0.2 mM and 0.5 mg/ml, respectively. The solution in the test tube was incubated at 37 C for 24 hr.

Mixtures of 1 mM 12-KOA and 50 mM amino acids in 0.1 M phosphate buffer, pH 7.4, containing 20% ethanol were incubated at 37 C for 24 hr. After incubation, pH of each sample was adjusted to 7.4. The fluorescence intensity of the solution then was measured directly in a Hitachi 650 fluorospectrometer and calculated relative to that of quinine sulfate standard (0.2 mg/l of 0.1 N H_2SO_4, Ex 351 nm, Em 448 nm), which was taken as 100.

Quantitative Determination of 12-KOA

12-KOA was determined by gas chromatography with 12-KSA as internal standard. Mixtures after reaction of 12-KOA with amino acids were extracted three times with an equal volume of hexane. The extracts were combined, the hexane was evaporated under nitrogen, and 12-KOA was methylated with diazomethane. The amount of methyl ester in the sample then was measured in a Shimadzu gas chromatograph GC-6A equipped with a column (3 mm × 2 m) packed with 10% EGSP on 60–80 mesh chromosorb WAW. The column temperature was 195 C.

Polyacrylamide Gel Electrophoresis

After incubation for five days, the reaction products of BSA and 12-KOA were analyzed by polyacrylamide gel electrophoresis. Electrophoresis was carried out in 7.5% polyacrylamide gel with Tris-glycine buffer, pH 8.3, for five hr at 6.4 mA/cm under cooling at 18 C. The gel was stained with 0.1% Coomassie Brilliant Blue.

Preparation and Purification of a Main Fluorescent Product of 12-KOA Methyl Ester with Benzylamine

The reaction mixture containing 2.0 g of 12-KOA methyl ester and 1.08 g of benzylamine was stirred at 50 C. After 36 hr, the fluorescent products were separated by silicic acid column chromatography with chloroform/acetone (4:1, v/v) as solvent and purified by silica gel thin layer chromatography (TLC) with chloroform/acetone (1:1, v/v). The main product (more than 80%) was 12-oxo-10-octadecenoic acid methyl ester. The main fluorescent product (Ia in Fig. 4, Rf = 0.71) was obtained in a yield of 64 mg. The methyl ether (Ib) of Ia was prepared by mixing 3 ml of solution of 40 mg of Ia in methanol with 0.1 ml of 1 M HCl-methanol for five hr at room temperature. The solvent was evaporated and Ib was separated by silicic acid column chromatography with chloroform/acetone (94:4, v/v) as solvent.

Preparation of a Fluorescent Product of 12-KOA Methyl Ester with Glycine Methyl Ester

The fluorescent product (II) was prepared by mixing a solution of 1.0 g of 12-KOA methyl ester with 0.42 g of glycine methyl ester HCl in 2.5 ml of triethylamine for 12 hr at 60 C. The fluorescent products were separated and purified by silicic acid column chromatography and TLC with chloroform/acetone (3:1, v/v) and chloroform/acetone/methanol (6:3:1, v/v/v), respectively, as solvents. The Rf value of the main fluorescent product (II) was 0.34.

Characterization of Ia and II

The fluorescent products were characterized by infrared (IR), ultraviolet (UV), fluorescence, ^1H- and ^{13}C-nuclear magnetic resonance (NMR) and high- and low-resolution mass (MS) spectrometries. Electron impact ionization mass (EI-MS) spectra were measured with a JEOL JMS-D300 double-focusing mass spectrometer. Ionization was complete at 70 eV. High resolution MS spectra were obtained with perfluorokerosene as a standard. ^1H- and ^{13}C-NMR spectra were recorded in a JEOL JNM-FX200 spectrophotometer at 200 MHz and 50 MHz. The samples were dissolved in $CDCl_3$ with 1% tetramethylsilane as an internal reference. Uncorrected fluorescence spectrum of Ia was recorded with a Hitachi 650 instrument. IR spectra were determined in a JASCO DS-701G instrument by the liquid film method.

RESULTS AND DISCUSSION

On incubation of 12-KOA with BSA at pH 7.4, the fluorescence with an excitation maximum of 350 nm and an emission maximum at 420 nm increased markedly (Table 1). The spectrum was similar to that reported for the reaction product of peroxidized linoleic acid with BSA (13).

To obtain further insight into the reaction, we examined the fluorescence of reaction mixtures of 12-KOA and various amino acids. As shown in Figure 1, except in the case of

TABLE 1

Fluorescent Formation by the Reaction of 12-KOA with Amino Acids and Protein and Its Inhibition by SH-Amino Acids

	Ex (nm)	Em (nm)	Fluorescent intensity
Histidine	339	413	206.6
Arginine	319	383	108.0
Lysine	349	419	167.5
Glycine	342	414	135.8
Glutathione	323	423	15.0
N-Acetyl-glycine	322	401	17.1
N-Acetyl-cysteine	313	351	<1.0
Glycine + N-acetyl-cysteine	327	369	1.2
Glycine + glutathione	335	425	26.1
Lysine + N-acetyl-cysteine	340	419	45.0
BSA	350	420	153.0
Polylysine	334	408	225.0

Concentration of each amino acid was 50 mM.

Concentrations of BSA and polylysine were 3 mg/ml and 0.5 mg/ml, respectively.

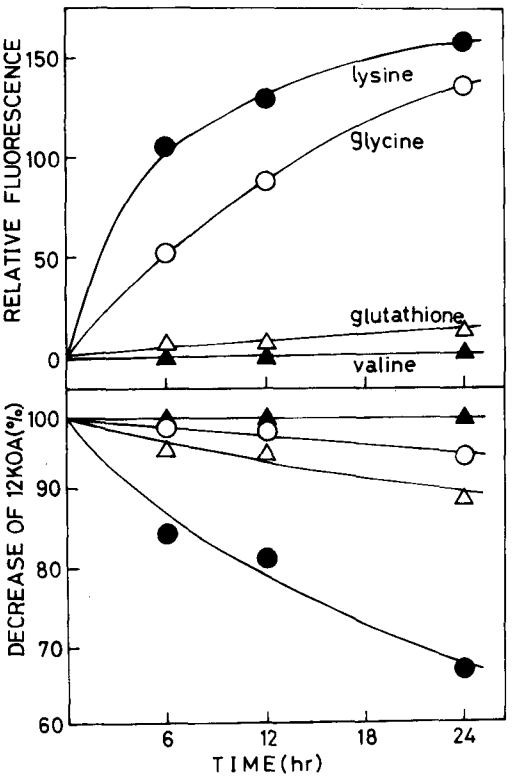

FIG. 1. Time courses of formation of fluorescence and 12-KOA consumption during its reacting with lysine (●), glycine (○), valine (▲) and glutathione (△). Reaction mixture containing 1 mM 12 KOA, 50 mM amino acids, 20% ethanol and 0.1 M phosphate buffer, pH 7.4, was incubated at 37 C. The fluorescent intensity of 0.2 mg of quinine sulfate/l of 0.1 N H_2SO_4 (Ex 351 nm, Em 448 nm) was set at 100.

glutathione, the increase in fluorescence intensity was proportional to the amount of 12-KOA consumed, in the order lysine > glycine > valine = 0. The mixture with glutathione showed little fluorescence, though significant 12-KOA was consumed during the reaction. Table 1 shows that besides glycine, only the basic amino acids histidine, lysine and arginine gave strongly fluorescent products. Acidic and neutral amino acids examined (asparatic acid, glutamic acid, glutamine, alanine, isoleusine, valine, serine, threonine, phenylalanine, proline and methionine) did not react with 12-KOA, resulting in little fluorescence (data not shown). In the reaction of peroxidized lipid also, only basic amino acids produce fluorescent products (13). Little fluorescence was observed with N-acetyl-glycine, but glycine gave strong fluorescence, indicating that a free amino group was essential for formation of a fluorescent product. The amino groups at the α-position in acidic and neutral amino acids other than glycine may be unable to react with 12-KOA due to steric hindrance of side chains. These results indicate that one of the reaction sites of BSA responsible for fluorescence is free amino groups of lysine and arginine moieties in the protein. This conclusion was supported by the result that polylysine, which contains only free amino groups in the molecule, gave strong fluorescence by reaction with 12-KOA (Table 1).

Figure 2 shows the results obtained on polyacrylamide gel electrophoresis of BSA with and without treatment with 12-KOA or ricinoleic acid. The higher mobilities of the two components of 12-KOA-treated BSA than those of untreated or ricinoleic acid-treated BSA indicate the increase in acidic properties of the components. This finding suggests that basic amino acid residues, such as lysine, arginine and histidine, in BSA were modified by 12-KOA.

Glutathione or N-acetyl-cysteine, which reacted with 12-KOA but produced no fluorescent products, strongly inhibited formation of fluorescent products on reaction of glycine or lysine with 12-KOA (Table 1). These results suggest that a thiol group is more reactive than an amino group with 12-KOA.

The formation of a fluorescent product on reaction of 12-KOA with glycine was investi-

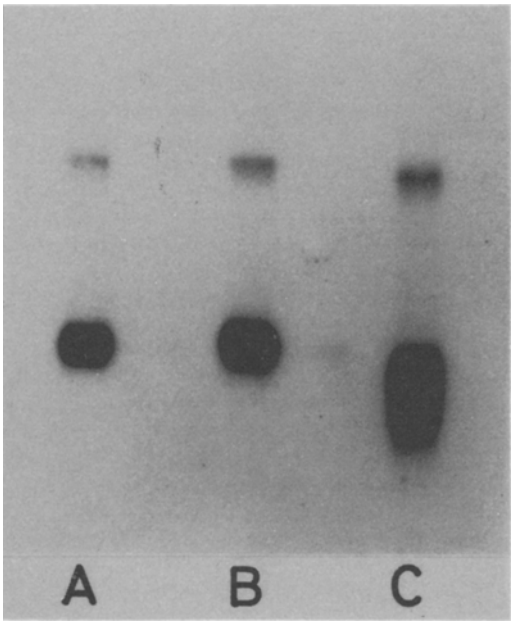

FIG. 2. Polyacrylamide disk gel electrophoresis of reaction products of BSA with 12-KOA. A, BSA; B, BSA + ricinoleic acid; C, BSA + 12-KOA. Fluorescent intensity of reaction products of 12-KOA with BSA was 328 (Ex 338 nm, Em 411 nm) when the intensity of 0.2 mg of quinine sulfate/l of 0.1 N H_2SO_4 was set at 100.

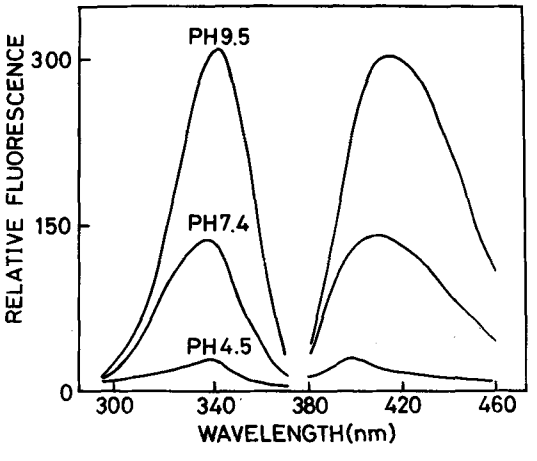

FIG. 3. pH-Dependent fluorescence formation during reaction of 12-KOA with glycine. Experimental conditions were the same as for Fig. 1, except that the incubation mixture at pH 4.5 contained 40% ethanol. Each pH was adjusted with NaOH or HCl. After incubation at the indicated pH values for 24 hr at 37 C, samples were adjusted to pH 7.4 and then fluorescence was measured.

gated at pH values of 4.5, 7.4 and 9.5. As shown in Figure 3, the fluorescence increased with increase of pH, suggesting that a NH_2, not NH_3^+, group reacted with 12-KOA.

We next examined the relationship between the structure of fatty acids and their ability to form a fluorescent product. As shown in Table 2, fatty acids with structures analogous to that of 12-KOA (β,γ-unsaturated keto acid), such as 12-KCA (α,β-unsaturated keto acid), 12-KSA (saturated keto acid), ricinoleic acid (β,γ-unsaturated hydroxy acid) and oleic acid, were tested, but none formed a fluorescent product when incubated with glycine. Thus, a β,γ-unsaturated carbonyl structure appeared essential for production of the fluorescent product. It is very interesting that 12-KOA had the highest activity to form a fluorescent product, since the potency of 12-KOA in induction of encephalomalacia in chicks has been reported also to be higher than those of 12-KCA and 12-KSA (16). Hexenal, another possible model of secondary products of peroxidized lipids (26,27), also produced a fluorescent product on reaction with glycine, but this had less fluorescence than the product of 12-KOA with glycine.

For elucidation of the structure of the fluorescent products, we prepared the Ia, a main fluorescent product, formed by a reaction of 12-KOA methyl ester with benzylamine, an aliphatic primary amine. As shown in Figure 5, Ia shows a fluorescence spectrum with an excitation maximum at 340 nm and an emission maximum at 410 nm, unlike the malondialdehyde-protein complex, which shows values of 395–405 nm and 460–465 nm, respectively (15). The spectrum of Ia was similar to that of the reaction product of oxidized lipid and protein (12).

The molecular weight of compound Ia was determined as 429.2881 ($C_{26}H_{39}N_1O_4$) by high

TABLE 2

Fluorescence Produced by Reactions of 12-KOA Analogs with Glycine

Fatty acid	Ex (nm)	Em (nm)	Fluorescent intensity
12-KOA	342	414	132.0
12-KCA[a]	340	408	47.7
12-KSA	308	368	<1.0
Ricinoleic acid	313	373	<1.0
Oleic acid	305	379	<1.0
Linoleic acid	314	373	<1.0
Hexenal	326	411	41.6
Hexanal	340	411	13.7

[a]Contaminated with 30% 12-KOA.

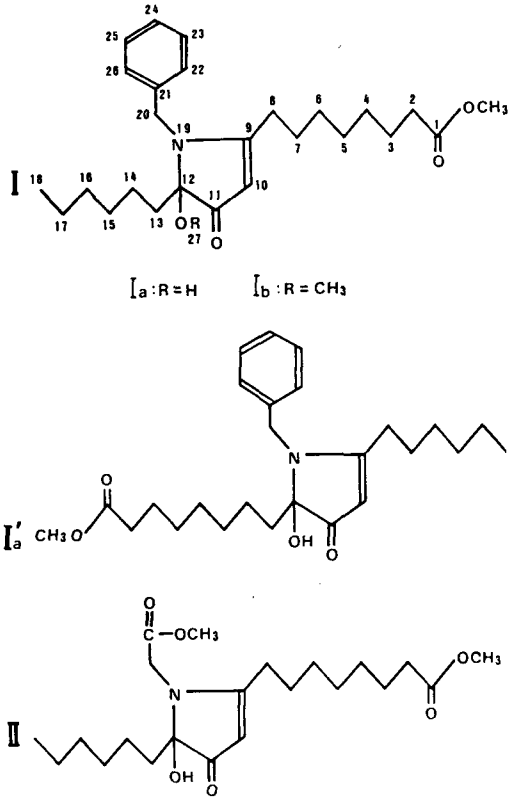

FIG. 4. Numerical key and structures of main fluorescent products and derivatives of the 12-KOA methyl ester and benzylamine and glycine methyl ester.

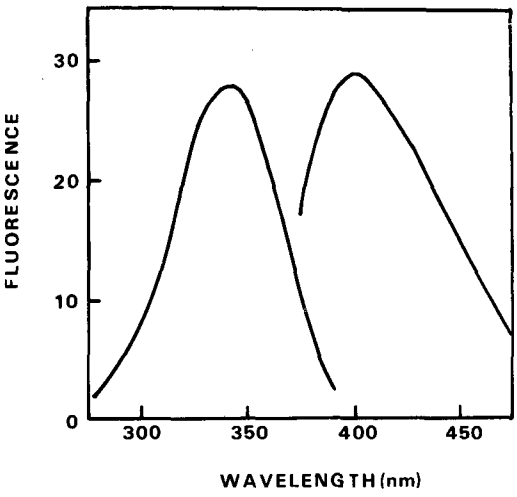

FIG. 5. Fluorescence spectrum of Ia in methanol. The concentration of Ia was 28.8 μM (0.295 absorbance at 340 nm). The fluorescent intensity of 0.5 mg of quinine sulfate/l of 0.1 N H_2SO_4 was set at 100 (Ex 340 nm, Em 408 nm).

resolution MS spectrometry. This value indicates the introduction of one oxygen atom as well as a benzyl amino group into the original β,γ-unsaturated ketone. Other fragment ions m/z 411 (M-H_2O), 398 (M-OCH_3), 338 (M-91), 316 (M-113) and 91 were also characteristic of Ia (Fig. 6).

Data on ^{1}H-NMR analysis shown in Table 3 indicate the presence of a methoxy, a terminal methyl and a benzylamino group and twelve methylene groups, of which two (corresponding to C_2 and C_8 of Ia, Fig. 4) are observed in a lower field than other methylene groups of Ia.

The absorptions at 1650 cm^{-1} in the IR spectrum and at 340 nm (ε = 10240) in the UV spectrum indicate the presence of an α,β-unsaturated ketone group. In the ^{1}H-NMR spectrum, the olefinic proton resonance is observed as a proton singlet at a higher field than that of normal olefinic proton conjugated with a ketone group. This position (δ4.95) is consistent with the conjugation of the enone group with some hetero atoms.

The other oxygen atom of the molecule is attributed to the tertiary hydroxyl group on the basis of the ^{1}H-NMR (δ4.48, singlet, exchangeable with D_2O) and the IR spectra (3240 cm^{-1}).

From these spectroscopic data and the molecular formula, in which one ring structure or one unsaturated bond other than that described above is necessary, the formula Ia or Ia′ is possible for the fluorescent compound. The latter formula (Ia), however, is not compatible with the fragmentation pattern of the MS spectrum in Figure 6. The ^{13}C-NMR data (Table 4) are fully consistent with formula Ia.

For confirmation of the aminol moiety (C_{12}-N_{19} in Fig. 4) of the molecules, compound Ia was treated with 1 M HCl in methanol at room temperature to give the methyl ester (Ib) in good yield as described in the Experimental section. The spectroscopic data for Ib were very similar to those for Ia except that a methoxy group (δ3.05, singlet) was observed in place of a hydroxyl group (δ4.48) in the ^{1}H-NMR spectrum. The EI-MS spectrum of Ib also showed the ion m/z 443 (M^+), 428 (M-CH_3), 412 (M-OCH_3), 358 (M-85), 352 (M-91) and 91 (Fig. 6). The IR spectrum of Ib showed less absorption at 3420 cm^{-1} than that of Ia, indicating loss of a hydroxyl group.

Addition of trifluoroacetic acid to the chloroform solution of Ia resulted in profound change of the UV spectrum. As shown in Figure 7, a new absorption peak appeared at 273 nm with decrease in absorption at 340 nm, suggesting ring opening of the aminol moiety (Fig. 8). The spectra had two isobestic points at 315 and 370 nm, and the change induced by

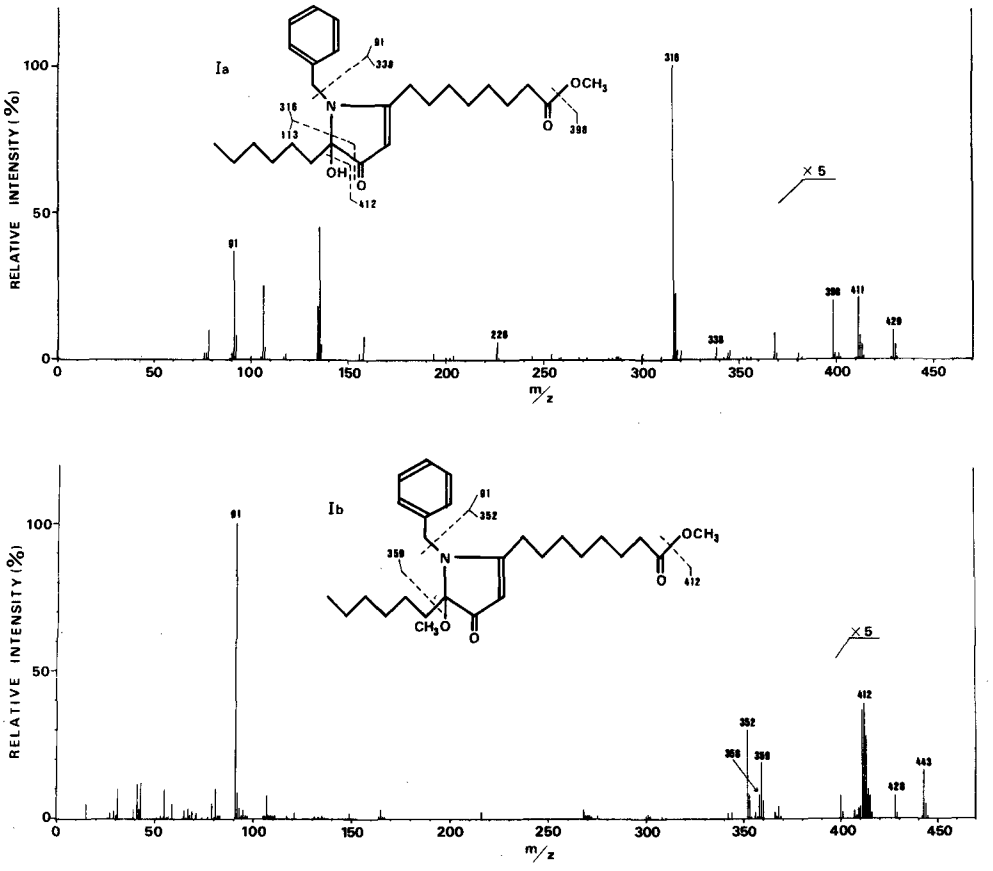

FIG. 6. Electron impact ionization mass spectra of Ia and Ib.

TABLE 3

^{1}H-NMR Spectra of the Fluorescent Product (Ia) and Its Methyl Ether (Ib)

ppm			Coupling constant	No. of	
Ia	Ib	Multiplicity[a]	(Hz)	protons	Assignment
7.32	7.27	s		5	Ar
4.95	5.05	s		1	H-10
4.62		d	J = 17	1	H-20
4.49		d	J = 17	1	H-20
	4.46	s		1	H-20
4.48		br, s		1	OH
3.66	3.67	s		3	OCH_3
	3.05	s		3	OCH_3 (H-27)
2.28		t	J = 7	4	H-2, H-8
	2.49	t	J = 7	2	H-8
	2.30	t	J = 7	2	H-2
1-2	1-2	br		18	H-3-7, H-14-17
0.84	0.81	t	J = 6	3	H-18

[a]Multiplicity: br = broad, s = singlet, d = doublet, t = triplet.

TABLE 4

^{13}C-NMR Spectra of the Fluorescent Product (Ia) and Its Methyl Ether (Ib)

ppm			
Ia	Ib	Multiplicity[a]	Assignment
200.72	198.00	s	C-11
182.29	182.35	s	C-9
174.09	174.06	s	C-1
138.02	137.44	s	C-21
128.68	128.77	d	C-23, C-25
127.43	127.72	d	C-24
127.31	127.57	d	C-22, C-26
94.96	97.35	d	C-10
91.39	96.15	s	C-12
	51.92[b]	q	C-27
51.42	51.45[b]	q	OCH_3
45.40	45.11	t	C-20
36.18	35.18	t	
33.99	33.99	t	
31.62	31.54	t	
29.17	29.17	t	
29.05	29.05	t	
28.96	28.99	t	
28.88	28.90	t	C-2-8, C-14-17
28.79		t	
26.60	26.92	t	
24.79	24.82	t	
22.71	22.48	t	
22.51	22.19	t	
14.02	14.05	q	C-18

[a]Multiplicity: s = singlet, d = doublet, t = triplet, q = quartet.

[b]May be reverse.

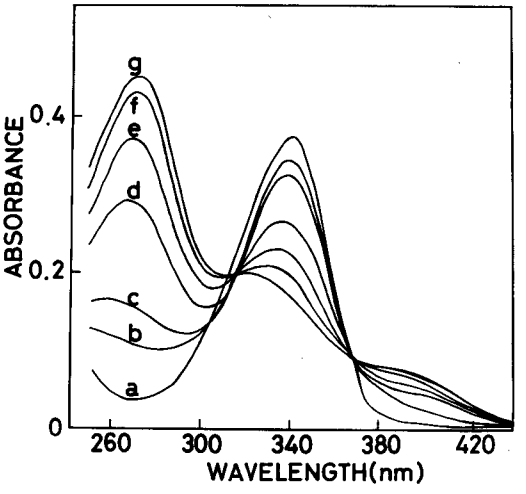

FIG. 7. Ultraviolet spectra of Ia in methanol with different concentrations of trifluoroacetic acid. Letters indicate concentrations (v/v) of trifluoroacetic acid of a, 0%; b, 0.3%; c, 0.6%; d, 0.9%; e, 2.7%; f, 5.4% and g, 8.1%.

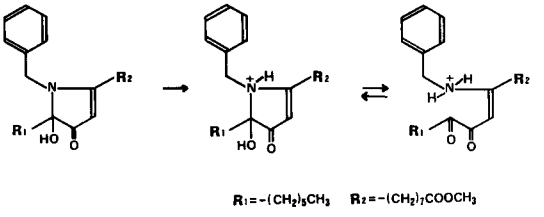

FIG. 8. Reversible structural change of Ia.

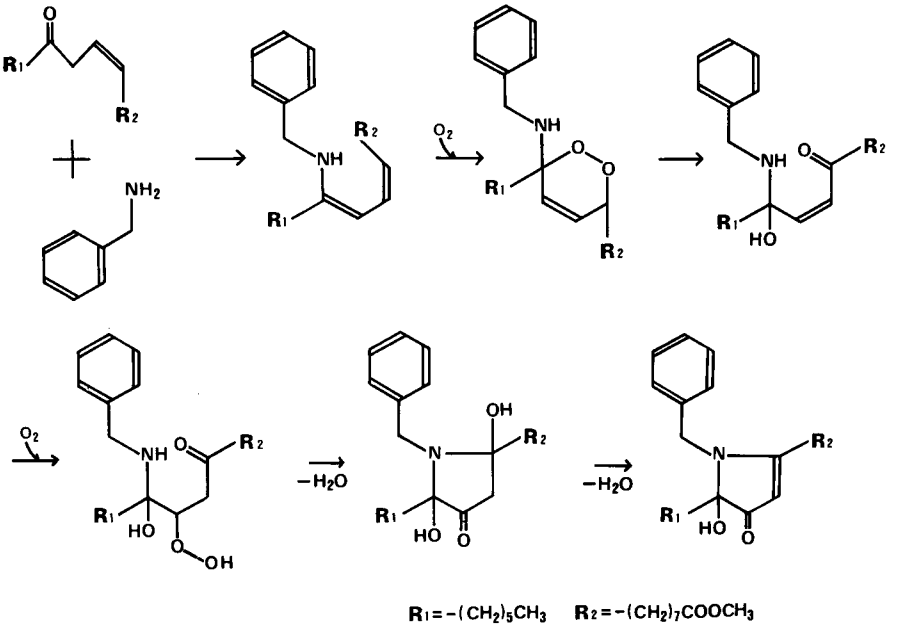

FIG. 9. Proposed pathway for formation of addition products. The fatty acid structure is abbreviated.

trifluoroacetic acid was reversed by addition of KOH. These findings support the proposed structure Ia.

The main fluorescent product (compound II, Fig. 4) of the glycine methyl ester with 12-KOA methyl ester was characterized by its EI-MS spectrum, having ion peaks m/z 411 (M^+, $C_{22}H_{37}N_1O_6$), 393 (M-H_2O, $C_{22}H_{35}N_1O_5$), 380 (M-OCH_3, $C_{21}H_{34}N_1O_5$), 352 (M-$COCH_3$, $C_{20}H_{34}N_1O_4$) and 298 (M-113, $C_{15}H_{24}N_1O_5$).

A possible pathway for formation of Ia is shown in Figure 9. The reaction of 12-KOA and benzylamine or glycine involves nucleophilic attack on the β,γ-unsaturated system of 12-KOA, which is followed by loss of water to yield the conjugated Shiff base. Oxygenation of the diene moiety followed by cleavage of the resulting peroxy ring gives the α,β-unsaturated ketone. Further oxygenation and dehydrations should give Ia. Other lipid peroxidation by-products having β,γ-unsaturated ketone, such as 12-oxo-9-dodecenoate (24), and proteins may undergo similar reactions.

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Short Term Essential Fatty Acid Deficiency in Rats. Influence of Dietary Carbohydrates

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ABSTRACT

The effects of long term (8–14 wk) essential fatty acid (EFA)-deprived diets in rats are well documented. In the present study, we compared, in weanling rats, the effect of a short term (two wk) hydrogenated coconut oil, EFA-deprived, diet (D) with that of a corn oil, EFA-adequate, diet (A), using either sucrose (SU) or starch (ST) as carbohydrate. After two wk, rats fed the sucrose/hydrogenated coconut oil diet developed some characteristic features of EFA deprivation: slower growth rate, decreases in linoleic and arachidonic acid of plasma phospholipids and an increase in n-9 eicosatrienoic acid of plasma phospholipids. When rats ate the starch/hydrogenated coconut oil diet, there was a similar decrease in linoleic acid of plasma phospholipids, but only a small effect on growth rate and no change in the arachidonic acid content of plasma phospholipids. EFA deprivation and sucrose had opposite effects on plasma triglyceride (TG) levels: deprivation induced a decrease, whereas the sucrose induced an increase in very low density lipoprotein (VLDL) triglycerides. The observed decrease in plasma triglyceride during EFA deprivation might result from an activation of lipoprotein lipase during the early stages of deprivation.
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INTRODUCTION

EFA-deprived diets lead to numerous changes in rat lipid metabolism when administered for eight wk or longer. In the liver, they increase fatty acid synthesis and TG and cholesterol ester (CE) deposition; in plasma they decrease TG and cholesterol concentrations (1–3). In addition, there are major alterations in the fatty acid composition of various plasma and tissue lipids (for review, see Holman [4]), revealing a marked decrease in the n-6 fatty acid group (mainly linoleic and arachidonic acids) associated with an increase in the n-9 fatty acid group (oleic and eicosatrienoic acids). Supplying the diet with hydrogenated fats or saturated fatty acids exaggerated the EFA deprivation (5,6). Given for shorter periods (up to 10 days), a saturated fat diet decreased primarily linoleic acid, with little effect on plasma arachidonic acid content (7). Rat lipid metabolism is dependent upon dietary EFA and dietary carbohydrate supply as well (8). Thus, sucrose exerted a higher stimulative effect on hepatic fatty acid synthesis (9,10) and on the rate of TG secretion than either dietary glucose or starch (11). The hypertriglyceridemic response to sucrose (12) likely is related to the effects of its fructose moiety on several hepatic enzymes (9,13,14), fructose being catabolized preferentially in the liver whereas glucose is a major nutrient for extrahepatic tissues (15).

Little is known from previous work on the early stages of EFA deprivation in young rats. Moreover, the influence of dietary sucrose compared to dietary starch under those deprived conditions was not studied, despite the fact that sucrose is a major dietary carbohydrate. To investigate the difference between these carbohydrates, we undertook a study using weanling rats fed for 15 days a diet containing either sucrose or starch as carbohydrate component and corn oil or hydrogenated coconut oil as lipid component. Under these experimental conditions, we have determined plasma cholesterol, TG and phospholipid concentrations; the lipid composition of plasma lipoproteins; the fatty acid pattern of plasma phospholipids, and the rate of TG secretion.

MATERIAL AND METHODS

Animals and Diets

Male weanling Wistar rats (21 days old) were fed for two wk one of four diets (10 rats per diet). All diets were isocaloric, based on the following caloric values: 16.7 KJ/g for dietary protein and carbohydrate, 37.6 KJ/g for dietary fat (Table 1). Vitamin and salt mixtures were according to the recommendations of the ad hoc Committee on Standards for Nutritional Studies of the American Institute of Nutrition

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TABLE 1

Composition of the Diets

	Corn oil (g/100 g [dry weight])		Hydrogenated coconut oil (g/100 g [dry weight])	
	A + SU[a]	A + ST[a]	D + SU[a]	D + ST[a]
Corn oil[b]	5.0	5.0	—	—
Hydrogenated coconut oil[c]	—	—	5.0	5.0
Casein	20	20	—	—
Delipidized[d] casein	—	—	20	20
Sucrose[e]	70.5	—	70.5	—
Starch[e]	—	70.5	—	70.5
Mineral mixture[f]	3.5	3.5	3.5	3.5
Vitamin mixture[g]	1.0	1.0	1.0	1.0
Linoleic acid[h]	2.7	2.7	0.001	0.015
Linolenic acid[h]	0.3 (6.6%)	0.3 (6.6%)	— (0.002%)	0.001 (0.035%)

[a]A = EFA adequate; D = EFA-deprived; SU = sucrose; ST = starch.

[b]Composition of corn oil by weight was: 10% glycerol, 90% fatty acids (16:0 = 11.7; 18:0 = 2.3; 18:1 = 25:3; 18:2 n-6 = 60.1; 18:3 n-3 = 0.7).

[c]Composition of hydrogenated coconut oil by weight was: 24% glycerol, 76% fatty acids (8:0 = 2.8; 10:0 = 5.2; 12:0 = 51.9; 14:0 = 17.5; 16:0 = 9.6; 18:0 = 11.8; 18:1 = 0.8).

[d]From USBC, Cleveland, OH 44128.

[e]Fatty acid-free from INRA-Theix, Beaumont, France.

[f]Percent composition: calcium phosphate, dibasic = 50; sodium chloride = 7.4; 2.4; manganous carbonate = 0.35; ferric citrate = 0.6; zinc carbonate = 0.16; cupric carbonate = 0.03; potassium iodate = 0.001; sodium selenite = 0.001; chromium potassium sulfate = 0.055; carbohydrate defined to make 100%.

[g]1% of this Mixture in the diet provides per kg of diet: thiamine, HCl—6.0 mg; riboflavin, 6.0 mg; pyridoxine, HCl (vitamin B6)—7.0 mg; nicotinic acid, 30.0 mg; calcium panthotenate, 16.0 mg; folic acid, 2.0 mg; biotin, 0.2 mg; cyanocobalamine = 10 μg; vitamin A = 4000 IU; vitamin D = 1000 IU; vitamin E, 50 IU; vitamin K, 50 μg.

[h]Values in parentheses refer to % energy in the diet. Results of GLC analysis of the diets were (in weight %): A + SU and A + ST = 18:2 n-6 = 60.1; 18:3 n-3 = 0.7; D + SU = 18:2 n-6 = 0.02; D + ST = 18:2 n-6 = 0.39; 18:3 n-3 = 0.03.

(16). The carbohydrate source was either SU or ST provided by INRA-Theix (Beaumont, France). Diet A included corn oil as lipid source and diet D included hydrogenated coconut oil (INRA-Theix, Beaumont, France). Fresh food and drinking water were provided daily and were available ad libitum. Food intake was determined daily. All groups ate nearly the same quantity of food (12 ± 1 g/day) without any significant difference among them.

Experimental Procedure

Rats were fasted 8–10 hr before blood collection. They were anesthetized between 9 and 9:30 a.m. to avoid circadian variations. A control group of 10 animals was killed prior to the feeding period to determine plasma lipid prior to the diet establishment. At day 14 after beginning the diets, blood was withdrawn by aortic puncture and quickly centrifuged. Plasma samples were used immediately for further lipoprotein separation and lipid analysis. Before slaughter, rats were weighed and plasma volumes (PV) were calculated according to Wang and Hegsted: PV = (body weight × 0.041) − 1.59 (17).

Lipoprotein Fractionation

Plasma samples (1 ml) were submitted to a two-step ultracentrifugation procedure using a SW 41 rotor. The first step isolated chylomicrons (18); the second was performed on the bottom fraction (3 ml) by using the density gradient described by Glangeaud-Freudenthal et al. (19) and lasted for 26 hr at 196,000 g. Fractions of 0.5 ml were sampled automatically and protein content was monitored using a continuous flow photometer (Isco) with a 254 nm filter. They were pooled in three lipoprotein classes according to the following densities: $d < 1.006$ for VLDL; $1.006 < d < 1.063$ for low density lipoprotein (LDL); $1.063 < d < 1.21$ for high density lipoprotein (HDL). Protein determination on each fraction was made according to Lowry (20).

Lipid Analysis

Triacylglycerol, total cholesterol and phospholipid concentrations were analyzed by enzymatic methods using Biomerieux kits. Plasma lipids were extracted according to Bligh and Dyer (21) and were separated by thin layer

chromatography according to Skipski (22). The corresponding spots were scraped, hydrolyzed with 0.5 N KOH-CH_3 OH and transmethylated with BF_3-CH_3OH at 60 C under dry nitrogen (23). Fatty acids were separated by gas liquid chromatography (GLC) (using an 82′ glass capillary column packed with Carbowax 20 M) and operated over a range from 150 to 200 C (linear increase in temperature 2 C/min). Aliquots from the four diets were extracted extensively with chloroform/methanol (2:1, v/v) and were hydrolyzed as described above. The residues then were hydrolyzed with hydrochloric acid under nitrogen for 24 hr in sealed vials and extracted with hexane. The extracts were pooled and submitted to GLC using heptadecanoic acid as internal standard.

Determination of VLDL Secretion Rate

Rats were injected intravenously with a solution of Triton WR 1339 in saline (0.25 mg/g body weight in 0.5 ml saline) according to Bird and Williams (11). Four hr later, blood samples were collected by aortic puncture and plasma TG were measured. The Triton technique provides a reasonable estimate of the rate of hepatic TG secretion. The fractional rate constant (FRC) for plasma TG turnover was estimated by dividing the rate of TG secretion (mM/min/100 g body weight) by plasma TG pool size (μM/100 g body weight).

Statistical Analysis

Results shown are means ± standard deviation. Comparisons between groups were made using the Wilcoxon-Mann-Withney test.

RESULTS

Rats from the two EFA-deprived groups did not develop any typical clinical symptoms following two wk of deprivation. The only significant difference between the deprived and adequately fed groups was a decrease in body weight in the deprived groups. This effect on body weight was more marked when the carbohydrate source was sucrose (Table 2).

No significant differences in plasma phospholipid concentrations were observed among the groups (Table 3). In the EFA-deprived rats (D + SU), sucrose resulted in little increase in plasma cholesterol concentration (Table 3). In rats fed the sucrose/EFA-adequate diet (A + SU), plasma TG concentration was two-fold higher than in the control group (i.e., before the experimental diet). Compared to the EFA-adequate diet, the EFA-deprived diet had a lowering effect on plasma TG content, irrespective of the type of carbohydrate (Table 3). Analysis of VLDL isolated from plasma of each rat revealed a much lower proportion of TG in EFA-deprived rats on both carbohydrate diets (Table 4). However, this decrease in VLDL-TG was more striking with dietary starch (from 45% to 6% of the total VLDL lipid) (Table 4).

A slight TG decrease also was observed in the LDL fraction (data not shown). The reduction in VLDL-TG was associated with an increased proportion of VLDL cholesterol (44% vs 24% for SU and 86% vs 48% for ST) (Table 4).

TABLE 2

Effects of Diets on Body Weight of Rats Fed Corn Oil or Hydrogenated Coconut Oil for Two Weeks

	Corn oil (A) (body weight, g)	Hydrogenated coconut oil (body weight, g)
Sucrose (SU)	94.6 ± 6.1[a,*]	64.9 ± 6.3[b]
Starch (ST)	97.2 ± 6.6[c]	89.7 ± 8.4[d]

Before experiment, weanling rat weight = 43.2 ± 5.3 g. Results are given as means ± SD for 10 rats/group. There were no significant differences in the weights of rats fed corn oil with sucrose or starch.

*The p values for the differences observed were: between a and b, $p < 0.005$; between b and d, $p < 0.005$; between c and d, $p < 0.05$.

TABLE 3

Effect of Diets on Plasma Lipid Concentrations

Lipids	Before diet (control) (mM)	A + SU (mM)	D + SU (mM)	A + ST (mM)	D + ST (mM)
Phospholipids	1.53 ± 0.26	1.98 ± 0.14	1.70 ± 0.63	1.28 ± 0.38	1.30 ± 0.13
Total cholesterol	2.10 ± 0.17[d,*]	2.22 ± 0.11[d]	2.77 ± 0.50[e]	2.35 ± 0.15	2.25 ± 0.30
Triglycerides	0.88 ± 0.18[a]	2.11 ± 0.28[b]	1.14 ± 0.50[a]	1.0 ± 0.20[a]	0.31 ± 0.03[c]

See Table 1 for group definition.

*p Values: a vs b, b vs c, a vs c: $p < 0.001$; d vs e: $p < 0.005$. Other values were not significantly different.

TABLE 4

Lipid Composition of Serum VLDL

Lipids	A + SU (mM)	D + SU (mM)	A + ST (mM)	D + ST (mM)
Phospholipids	$0.72 \pm 0.05^{f,*}$ (22%)	0.71 ± 0.25^{f} (24%)	0.08 ± 0.02^{g} (7%)	0.15 ± 0.05^{g} (8%)
Total cholesterol	0.82 ± 0.04^{d} (24%)	1.37 ± 0.27^{e} (44%)	0.61 ± 0.03^{d} (48%)	1.56 ± 0.17^{e} (86%)
Triglycerides	1.84 ± 0.27^{a} (54%)	1.01 ± 0.41^{b} (32%)	0.57 ± 0.07^{c} (45%)	0.11 ± 0.01^{c} (6%)
% of total plasma TG	87	88	57	35

See Table 1 for group definition. VLDL were prepared from the sera of 10 rats for each group.
Numbers in brackets display the percentage composition.
*p Values: a vs b, d vs e = $p < 0.005$; a vs c, f vs g = $p < 0.001$.

TABLE 5

Fatty Acid Profile of Plasma Phospholipids

	Before diet	A + SU	D + SU	A + ST	D + ST
16:0	27.4 ± 0.3	22.8 ± 1.4	28.3 ± 2.5	24.7 ± 1.7	27.2 ± 1.6
16:1	0.9 ± 0.06	3.9 ± 0.4	8.3 ± 1.5	1.5 ± 0.5	4.5 ± 0.8
18:0	18.3 ± 0.2	13.4 ± 0.9	10.3 ± 1.0	10.6 ± 0.5	12.2 ± 0.6
18:1	5.5 ± 0.06	18.2 ± 0.6	36.6 ± 0.5	19.2 ± 0.7	25.1 ± 1.2
18:2n-6	23.9 ± 1.0	19.7 ± 1.1	5.9 ± 1.7	18.6 ± 0.9	7.7 ± 1.3
20:3n-6	1.5 ± 0.1	0.9 ± 0.1	0.1 ± 0.1	2.2 ± 0.2	2.1 ± 0.4
20:3n-9	0.2 ± 0.1	0.3 ± 0.2	3.5 ± 0.6	0.3 ± 0.1	1.4 ± 0.3
20:4n-6	20.3 ± 0.8	20.6 ± 1.5	7.0 ± 1.3	19.9 ± 1.0	19.2 ± 0.9
20:3n-9 / 20:4n-6	0.010	0.014	0.500	0.016	0.075

Results are given in mole %, n = 10 for each group.
See Table 1 for group definition.

TABLE 6

Rates of Triglyceride Secretion into Plasma

	A + SU	D + SU	A + ST	D + ST
Rate of TG secretion nmole/min 100 g body wt	$73.6 \pm 2.0^{a,*}$	88.5 ± 1.1^{a}	49.2 ± 3.1^{b}	43.6 ± 1.1^{b}
Plasma TG pool size[+] μmole/100 g body wt	4.99 ± 0.9^{c}	1.33 ± 0.2^{d}	2.48 ± 0.2^{c}	0.72 ± 0.01^{d}
Fractional rate constant[†]	0.015 ± 0.006^{g}	0.066 ± 0.003^{h}	0.019 ± 0.005^{g}	0.060 ± 0.003^{h}

For each group n = 10, see Table 1 for their definition.
*p Values: a vs b, c vs d, g vs h = $p < 0.001$.
[+]Pool size was calculated by multiplying plasma TG concentration by plasma volume.
[†]Fractional rate constant equals rate of secretion divided by pool size.

The fatty acid composition of plasma phospholipids confirmed the linoleic acid deprivation in both EFA-deprived groups (5.9 ± 1.7 vs 19.7 ± 1.1 for SU and 7.7 ± 1.3 vs 18.6 ± 0.9 for ST (Table 5). However, eicosatrienoic acid n-6 and arachidonic acid were reduced only with D + SU (Table 5). Conversely, the proportion of eicosatrienoic acid n-9 was higher with D + SU than with D + ST (Table 5). A similar observation was made for palmitoleic and oleic acids.

Since the liver is the major if not the only source of plasma TG in fasting animals, the post-Triton hypertriglyceridemia directly reflects the hepatic TG release into plasma and allows its measurement (9,24). TG analysis was

made before and four hr after a Triton WR 1339 injection into the same experimental group of rats fasted for 10 hr prior to the experiment.

Table 6 shows that in weanling rats raised for two wk on EFA-adequate diet, A + SU exerted a higher stimulating effect on hepatic TG release than did A + ST. The difference between the plasma TG pool size in either circumstance accounts for the lack of significant difference in the FRC (0.015 ± 0.006 vs 0.019 ± 0.005). The same is true for EFA-deprived diets (compare D + SU and D + ST).

The rate of hepatic TG secretion was not altered significantly by EFA-deprivation (88.5 nmol/min/100 g vs 73.6 for SU and 43.6 vs 49.2 for ST). Because the plasma TG pool size was lower in EFA-deprived than in EFA-adequate diets, the FRC for plasma TG turnover was considerably greater (four-fold in D + SU compared to A + SU groups and three-fold in D + ST compared to A + ST groups).

DISCUSSION

These results demonstrate the development of some characteristic features of EFA deprivation in weanling rats after a short period (two wk) on an EFA-deprived diet when the carbohydrate was sucrose.

According to Rayssiguier et al. (25), no modification of the growth rate occurred in rats fed EFA-adequate diets, whatever the carbohydrate supply (Table 2). In weanling rats fed sucrose, EFA deprivation yielded EFA deprivation, as assessed by the slower growth rate and the well known alteration in the fatty acid pattern of plasma phospholipids: decrease in linoleic acid, eicosatrienoic n-6 acid and arachidonic acid and rise in nonessential families (palmitoleic acid, oleic acid and eicosatrienoic n-9 acid) (Table 5). Adult rats fed the same diet for a shorter period did not develop such alterations. In the later case, there was only a decrease in linoleic acid content of plasma phospholipid (from 29.9% to 9.7%) and an increase in oleic and palmitoleic acids, without major changes in arachidonic acid and eicosatrienoic acids (7). Feeding the EFA-deprived diet for longer periods resulted in a strengthened effect, especially regarding the eicosatrienoic n-9 acid increase in both adult and weanling rats (1,11,26,27).

When starch was supplied in the diet instead of sucrose, there was only a slight but significant decrease in the growth rate due to EFA deprivation (Table 2). The linoleic acid content of plasma phospholipids dropped, with no significant alterations of eicosatrienoic and arachidonic acid (Table 5). The moderate effect of EFA deprivation in certain starch diets was attributed by Holman to a possible contamination of commercial starch by linoleic acid (4). We cannot rule out a somewhat higher linoleate contamination of the starch diet, compared to the sucrose diet, to explain the nonsignificant higher linoleic acid content of plasma phospholipids (7.7 ± 1.3% vs 5.9 ± 1.7%). However, this contamination was far below the adequate supply (0.035% in energy intake for D + ST and 0.002% for D + SU diet). It has been reported that replacement of dietary starch by sucrose reduced the proportion of linoleate in plasma, especially when linoleate intake was low (28). Furthermore, sucrose induced a higher rate of fatty acid synthesis than starch (9,10). Sucrose plus nonessential fatty acids have an additive effect on tritium incorporation from tritiated water into fatty acids in both fed and starved-refed rats (29). As a result of the increase in liver fatty acid synthesis, a more important competition with endogenous nonessential fatty acids might explain the rapid fall in arachidonic acid content in rats fed EFA-deprived diets.

The slower growth rate observed with D + SU could not be related to a decreased food intake. It is known that sucrose diets lead to a decrease in extrahepatic fatty acid synthesis (9,10). When sucrose and hydrogenated coconut oil were substituted for starch and corn oil, a subsequent change in mitochondrial lipids occurred, which may hamper the coupling between mitochondrial oxidation and ATP synthesis and lead to more fuel consumption by mitochondria (30,31). These factors might explain the reduced growth rate of weanling rats fed D + SU. When the EFA-deprived diet was given for longer periods of time (nine wk), no difference appeared between starch and sucrose, leading to the same EFA deprivation (11,27).

In weanling rats, triglyceridemia was slightly higher than in adult rats: 0.88 ± 0.18 mM in our experiments as compared with previously published data (0.58 to 0.80 mM) (9,11,15). When sucrose was supplied, triglyceridemia raised (2.11 ± 0.18 mM), whereas no change occurred with starch (Table 3) despite the same food intake. Our results were higher, however, than those previously reported by others using EFA-deprived diets for longer periods (> two mo) (9,11,32). This sucrose effect may result from a stimulation of the hepatic triglyceride synthesis by the fructose moiety as already reported (9,32–34). Actually, the fact that the rate of hepatic TG secretion was higher after sucrose feeding than starch feeding (73.6 ± 2.0 vs 49.2 ± 3.1 nmol/min/100 g of body weight)

emphasized this observation (Table 6). Thus, the hypertriglyceridemia might be due first to an increased secretion of TG by the liver. Moreover, in sucrose-fed rats, nearly 90% of plasma TG remained in VLDL in both EFA-adequate and EFA-deprived diets (Table 4). This might indicate that sucrose not only decreases peripheral fatty acid synthesis but also impairs fatty acid supply from VLDL to tissues, speeding up the EFA deprivation. Further experiments have to be performed to establish the actual target of the sucrose effect. One can hypothesize that both a stimulated TG secretion from the liver and a slower lipoprotein lipase activity could cooperate.

Triglyceridemia was lower in rats on EFA-deprived diets than those on EFA-adequate diets, whatever the carbohydrate source. This observation agrees with numerous studies on 10–14 wk deprivation (1,3,11,26), with the exception of Huang and Williams (8). In adult rats, short term deprivation did not reveal evidence for such alteration (7). Sucrose induced an increase in TG whereas EFA-deprivation induced a decrease. This effect was obvious in all lipoprotein fractions (data not shown), although slighter in lipoproteins other than VLDL. D + ST was most effective in decreasing VLDL-TG (Table 4). Cholesterol level seemed to compensate for this TG loss while phospholipids remained unchanged. Changes in the lipid composition of VLDL possibly result from alteration of some metabolic events taking place within the plasma, as evidenced by studies after long term deficiencies (2,11,35). Although the hepatic TG secretion essentially remained the same in both EFA-deprived and EFA-adequate rats (Table 6), the former group had a much lower plasma TG pool and a much higher FRC (three- to four-fold increase). Similar increases in the FRC were shown by Huang and Williams (8) and Bird and Williams (11) after 8–10 wk on a deprived diet, despite the fact that they have demonstrated a stimulation of TG secretion and less decrease of the pool of plasma TG. The steady or stimulated hepatic TG secretion under EFA deprivation seemed to contradict the significant drop in triglyceridemia. In D + ST, the TG loss is most striking in the VLDL fraction (−80%), while the overall plasma TG concentration dropped by 30% (Table 4). In this case, one can postulate an effect of the EFA deprivation on lipoprotein lipase activation (27) during the first two wk. Such stimulation could help to provide tissues with EFA, as reflected by the increase of nonesterified fatty acid in plasma during the course of EFA deprivation. This possibly contributes to the slower occurrence of EFA deprivation with D + ST than with D + SU. Indeed, D + SU might exacerbate the deprivation in the weanling rat through an alteration in lipoprotein lipase activity.

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Evidence for Diplasmalogen as the Major Component of Rabbit Sperm Phosphatidylethanolamine

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ABSTRACT

The question of whether diplasmalogens [1,2-di(O-1′-alkenyl) phosphatidyl derivatives] make up part of the plasmalogen component of cell phospholipids was examined using rabbit epididymal spermatozoa. These cells are readily obtained as a highly homogeneous suspension and long have been known to have high plasmalogen content. Phospholipids were determined by thin layer chromatography (TLC) with $CuSO_4$ staining. Plasmalogens were determined by hydrolysis of the phospholipids with TCA/HCl, followed by TLC and $CuSO_4$ staining. Ethanolamine derivatives were determined by ninhydrin. The phosphatidylethanolamine (PE) content of these cells was 29 ± 2 $\mu g/10^8$ cells, 90% of which was assayed as diplasmalogen and 10% as diacyl PE. No monoplasmalogen could be detected. The presence of diplasmalogen as the major component of PE was given further support from infrared and proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy, which showed the presence of O-1′-alkenyl substituents but near absence of O-acyl substituents. The phosphatidylcholine (PC) content of the cells was 104 ± 5 $\mu/10^8$ cells, of which 50% was monoplasmalogen with the 1′-alkenyl group on the 2 position of the glycerol moiety. No diplasmalogen was found in PC. The other phospholipids in rabbit sperm were phosphatidylglycerol (PG), cardiolipin (CL), sphingomyelin (SP) and lysophosphatidylcholine (LPC). Phosphatidylserine (PS) and phosphatidylinositol (PI) were present at the limits of detectability of the TLC method. None of these phospholipids contained plasmalogen. The PE component of rabbit sperm phospholipids appears to differ from that of the other cells in having the previously unreported diplasmalogen as its major constituent.

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INTRODUCTION

Alkenylether phospholipids (plasmalogens) are ubiquitous membrane constituents of virtually all animal cells and most anaerobic bacteria (1-13). They also are present in green pea and bean phospholipids (14). More recently, a substantial concentration of plasmalogens also has been found in macrophages (15) and polymorphonuclear leucocytes (16). There is general agreement that the overall composition of plasmalogens corresponds to glyceryl phosphoryl derivatives of choline or ethanolamine, which contain a fatty acyl group and an O-(1′-alkenyl) group as substituents on the other two hydroxyl groups. The proportions and position of the O-(1′-alkenyl) moiety vary depending on the source of the lipid material. In pig heart phospholipids, 75% was found at the 1 hydroxyl group and 25% at the 2 hydroxyl group of the glycerol moiety; in beef heart, 87% was at the 1 and 13% at the 2 position (17). In bovine brain, plasmalogens are described as being primarily 1-O-(1′-alkenyl) phosphoglycerides, 70% of which are present in the PE fraction (1). From the reports describing the plasmalogen composition found to date, a surprising observation emerged: the plasmalogens were all "monoplasmalogens," in the sense that they contained but one alkenylether group. This raised the question: do "diplasmalogens," phospholipids containing two alkenylether groups, exist in membranes? This was investigated using new methodology recently developed for analysis of plasmalogens (18) to examine the phospholipids of rabbit epididymal spermatozoa. Mammalian sperm long have been known to have a high content of plasmalogen (9). The preparation of rabbit epididymal sperm is a highly homogeneous population of dispersed cells (19) ideally suited to the methodology. In this paper, the existence and properties of diplasmalogens in the phospholipids of rabbit sperm are reported.

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MATERIALS AND METHODS

Reagents

The synthetic standards 1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine (DPPE), 1,2-dipalmitoyl-sn-glycerol-3-phosphocholine (DPPC), lysophosphatidylethanolamine (LPE), 1,2-dipalmitoyl-sn-glycerol-3-phosphoglycerol

(DPPG), CL, SP, PS, PI and LPC were obtained from Avanti Biochemicals (Birmingham, Alabama). PE from bovine brain (PEB) was obtained from Supelco Co. (Bellefonte, Pennsylvania). Glycerylphosphoethanolamine (GPE), glycerylphosphocholine (GPC) and phospholipase A_2 from snake venom (Naja Naja) (E.C. 3.1.1.4) were purchased from Sigma Chemical Co. (St. Louis, Missouri). The Schiff reagent was obtained from Accra-Lab Inc. (Bridgeport, New Jersey). Phospholipid standard solutions were prepared at concentrations between 0.5 and 2 mg/ml in chloroform/methanol (1:1, v/v). The purity of these standards was verified by TLC with different mobile phases. Solvents were EM Science chromatographic grade. Precoated Silica LK5 plates (250 μm thick) with preadsorbent zone of 500 μm thickness were obtained from Whatman Inc. (Clifton, New Jersey). Inorganic salts were from J.T. Baker (Phillipsburg, New Jersey) and the highest purity available.

Preparation of Spermatozoa

Rabbit epididymal spermatozoa were obtained from the caudae of excised epididymides of mature male New Zealand White rabbits by retrograde flushing through the vas deferens with 10 ml of a sodium Tris phosphate medium (NTP) of the following composition: 103 mM NaCl, 10 mM KCl, 12.5 mM NaH_2PO_4, 2.5 mM Na_2HPO_4, 3 mM $MgCl_2$, 20 mM Tris, 1.5 mM D-glucose, 0.4 mM EDTA, pH 7.4 (20). Sperm then were washed twice in this medium by centrifugation at 750 × g for 10 min. The final pellet was resuspended in medium NTP and adjusted to a cell concentration that ranged from 0.5–2 × 10^9 cells/ml.

Thin Layer Chromatography of Sperm Phospholipids

Whatman LK5 silica gel plates (20 × 20 cm; 250 μm thick with preadsorbent zone) were washed by continuous development overnight in chloroform/methanol (1:1, v/v). Those plates used for in situ chemical and enzymatic hydrolysis experiments were scored on a Schoeffel scoring device to give 1 cm lanes prior to the washing procedure. Development was carried out in standard size tanks.

Samples of sperm suspensions were applied directly to the preadsorbent zone in 125 μl aliquots as a thin band on the preadsorbent zone of unscored plates. Four plates were used in each determination. Synthetic or purified phospholipid standards were applied to the two edges of the plate for location of the unknown phospholipids. The plates were dried under a stream of warm air for 10 min. After drying, the layers were predeveloped three times in chloroform/methanol (1:1, v/v) to the interface of the preadsorbent zone. Between each predevelopment, the layers were air dried for a time sufficient to assure complete evaporation of the solvent. This procedure extracts the phospholipid from the sample and deposits it as a line on the starting point of the chromatogram (21). The mobile phase was chloroform/ethanol/triethylamine/water (30:34:30:8, v/v/v/v) (22). Development proceeded until the mobile phase reached 2 cm from the top of the plate; this usually required 1.5 hr. After development, the plates were dried and 1-inch strips from both edges of the plates, where the standards had been applied, were cut and dried in an oven at 170 C for two min to remove residual solvent. Half of the strips were sprayed with a 10% solution of $CuSO_4$ in 8% H_3PO_4; the other half were sprayed with a 0.2% solution of ninhydrin in acetone. The $CuSO_4$-sprayed plates were dried for five min at room temperature, heated in an oven at 100 C, and finally placed in an oven at 170 C for 10 min (21). Ninhydrin-sprayed plates were placed directly in an oven at 110 C for five min. These procedures gave optimal development of the chromatograms.

The developed chromatograms were scanned in a Kontes Fiber Optic Scanner (Model 800) using a 440 nm filter. A Hewlett-Packard 3390A integrator provided integration of the absorbance bands. The scanning was carried out in the transmission mode using double beam operation. DPPE, DPPC, LPE, LPC, PEB, LPEB, GPE and palmitic acid were used to obtain standard curves. Amounts of phospholipid between 0.5–5 μg gave linear standard curves, so procedures were adjusted so that the amounts applied to the plates were in this range. Amounts of the unknowns were interpolated directly from the standard curves and so are referred to the standards. Phospholipid phosphorous was calculated from these amounts, using the conversion factor 1 μg phospholipid = 0.04 μg P (9).

Spectroscopic Analysis of PE from Rabbit Sperm (PES) and PC from Rabbit Sperm (PCS)

PES and PCS were isolated directly from the plates by scraping off the spots located by $CuSO_4$ and ninhydrin staining. Phospholipid release from the silica gel was facilitated by adding a few drops of water, followed by 3 ml of chloroform/methanol. The suspension was homogenized with a Vortex mixer, then centrifuged at 600 × g for five min to remove silica. The liquid phase was separated and the silica

resuspended in 3 ml of chloroform/methanol (1:1, v/v) and centrifuged again at 600 × g for five min. The chloroform/methanol extract then was filtered through a Millipore 0.45 μm membrane to remove residual silica and dried under nitrogen until the solvent was completely removed. Recovery was 95%. Samples then were subjected to ^{1}H-NMR and infrared analysis.

Infrared spectrometry was performed by applying the lipid sample in chloroform at about 10 mg/ml as a film across the window area of a 200 mg KBr pellet and allowing the solvent to evaporate. The pellet was placed in a micro holder and scanning was carried out on a Perkin Elmer 421 infrared spectrometer. Interpretations of the spectra were based on the observations of Lammers et al. (23) and Meakings (24), and individual characteristics of functional groups were assigned as discussed by Bellamy (25), Chapman et al. (26), and Akutsu and Kyogoku (27).

^{1}H-NMR analysis was carried out in a Brucker WH-360 spectrometer. All samples were dissolved in $CDCl_3$. Chemical shifts are quoted in ppm downfield from the internal tetramethylsilane (TMS) standard and are accurate to within 0.005 ppm. The spectrometer was operated at 360 MHz. ^{1}H-NMR interpretations are based on the observations of Lammers et al. (23) and Hauser et al. (28).

Plasmalogen Identification by Acid Hydrolysis

Phospholipids from the sperm cells were subjected to acid hydrolysis, to which the alkenylether groups are very sensitive. Products are the corresponding glycerol and aldehyde derivatives. This provides a rapid method for plasmalogen identification (18). The reaction was carried out in situ by streaking 25 μl of each phospholipid at 2 mg/ml in chloroform/methanol (1:1, v/v) on the preadsorbent zone of scored LK5 plates in eight adjacent lanes. Then 25 μl of a mixture of 2% trichloroacetic acid and 8% hydrochloric acid (TCA/HCl) (1:1, v/v) was added to this zone of each lane. Plates remained at room temperature for 10 min to assure complete hydrolysis of the alkenylether group by the TCA/HCl mixture. Then two predevelopments and the final development of the chromatogram were carried out as described above. After development, the plates were dried and one strip was cut from each edge of the plate in such a way that two lanes would be on each strip. These strips were dried in an oven at 170 C for two min. One strip was sprayed with copper sulfate and the other with ninhydrin reagent, as described above. The solvent front area was sprayed with a solution of Schiff reagent (29) for aldehyde identification. Quantitation of the aldehyde was done only on the $CuSO_4$-sprayed plate. The products of hydrolysis then were scraped individually from the remaining unstained plate and subjected to either a second acid or enzymatic hydrolysis. The identical procedure was used for purified PEB.

Enzymatic Hydrolysis

Enzymatic hydrolysis was performed directly on the silica plate after separation of the phospholipids in exactly the same manner as for acid hydrolysis. After separation of the phospholipids in the eight lanes, 25 μl of phospholipase A_2 solution (5 mg/ml) in NTP with 2.5 mM $CaCl_2$ (30) was added to each lane. The plate remained at room temperature for 10 min to allow complete ester hydrolysis. The reaction mixture containing phospholipase A_2 remained at pH of 7.4 ± 0.2, as tested in control lanes, throughout the reaction period. The plates were dried, treated to develop the chromatogram, and stained with $CuSO_4$ and ninhydrin as described above.

RESULTS AND DISCUSSION

The different types of phospholipid present in rabbit epididymal spermatozoa, as determined by this TLC method with $CuSO_4$ staining, are shown in the densitometer tracing of Figure 1A. In some samples, peaks in the chromatogram corresponding to PS and PI were perceptible. These two components each must be present at no more than 1 μg/10^8 cells, which is the limit of detectability of this procedure. Sperm phospholipid content is listed in Table 1.

Treatment of sperm phospholipids with TCA/HCl reagent for determination of plasmalogen gave the result shown in the densitometer trace of the $CuSO_4$ stained plate in Figure 1B. The peak for PC decreased by about half, with a corresponding increase in LPC, as expected for a monoplasmalogen. The peak for PE was nearly eliminated, with no appearance of the corresponding lysophospholipid. None of the other phospholipids was affected by the TCA/HCl reagent within the limits of detectability, indicating that these do not contain plasmalogen.

The sperm PE, designated PES prior to any treatment, was isolated from the plate and rechromatographed. It gave a single sharp peak after $CuSO_4$ staining, as shown in Figure 2a. Treatment with the TCA/HCl reagent, followed

by $CuSO_4$ staining, yielded only a fatty aldehyde peak (Fig. 2b) and a small peak designated PES_1. The latter amounts to 10% of the original PES. The complementary treatment with phospholipase A_2 affected 10% of the original PES, yielding fatty acid and lysophospholipid ($LPES_2$) corresponding to this 10% (Fig. 2c). When the chromatogram corresponding to that of Figure 2b was stained with ninhydrin to identify the ethanolamine moiety of the PE molecule, only a small peak corresponding to PES_1 and a large peak corresponding to GPE were found (Fig. 2d). These results are consistent with PES containing 90% of the PE component as diplasmalogen with alkenylether moieties at the 1 and 2 positions and 10% PE as diacyl PE. The diacyl PE appears as PES_1 (Fig. 2b) while the diplasmalogen appears as PES_2 (Fig. 2c). The amount of monoplasmalogen must be at or

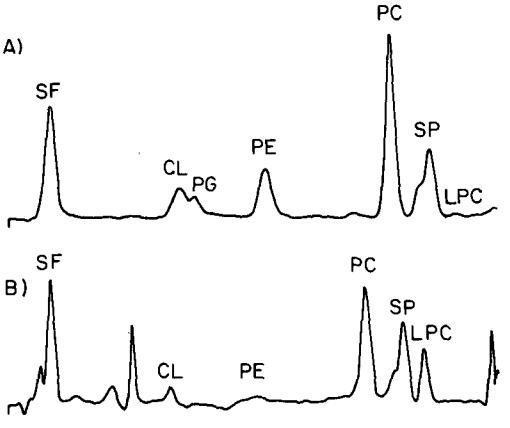


FIG. 1. Densitometric traces of TLC plates with rabbit epididymal sperm phospholipids separated and stained with $CuSO_4$. The unperturbed profile is shown in trace A. The components identified by the labeled bands to the right of the solvent front (SF) are: CL, cardiolipin; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SP, sphingomyelin, and LPC, lysophosphatidylcholine. The profile of the phospholipids which gave trace A and were treated with the TCA/HCl reagent is shown in trace B.

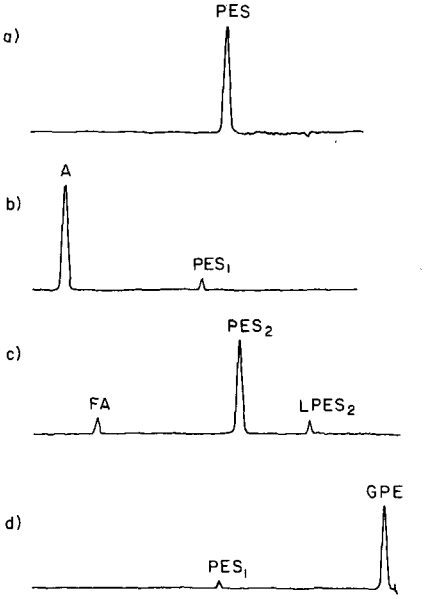


FIG. 2. Densitometric traces of TLC plates with isolated rabbit epididymal sperm phosphatidylethanolamine (PES) after various treatments, and stained with $CuSO_4$ or ninhydrin. The profile of untreated PES is shown in trace *a*; that of PES treated with the TCA/HCl reagent is shown in trace *b*. Staining was with $CuSO_4$. Band A in trace B was shown to be aldehyde with the Schiff reagent. The profile of PES treated with phospholipase A_2 and stained with $CuSO_4$ is shown in trace *c*; band FA is fatty acid. The profile obtained by treatment with the TCA/HCl reagent and stained with ninhydrin is shown in trace *d*; band GPE is glycerylphosphoethanolamine.

TABLE 1

Phospholipid Content[a] of Rabbit Epididymal Spermatozoa

Phospholipid	μg Phospholipid per 10^8 cells	μg P per 10^8 cells	% of total phospholipid
Phosphatidylglycerol	10 ± 1	0.39 ± 0.05	6.8
Cardiolipin	7 ± 1	0.27 ± 0.03	4.8
Phosphatidylethanolamine	20 ± 1	0.79 ± 0.06	13.9
Phosphatidylcholine	72 ± 3	2.7 ± 0.2	50.1
Sphingomyelin	32 ± 2	1.3 ± 0.1	22.7
Lysophosphatidylcholine	3 ± 1	0.12 ± 0.03	1.7
Total phospholipid	144	5.57	100

[a]Values are the means of 10 separate determinations on different samples; error limits are standard deviations.

below the unit of detection, since no lyso form was found in the chromatogram of Figure 2b, obtained by TCA/HCl treatment. Monoplasmalogens would be converted by this treatment to a lyso form of PE with the acyl moiety on either the 1 or 2 positions. There is also reasonable quantitative agreement between fatty aldehyde produced by TCA/HCl treatment (Fig. 2b) and PES_2 produced by phospholipase A_2 treatment (Fig. 2c). The same agreement is seen by comparison of PES_1 (Fig. 2b) and $LPES_2$ (Fig. 2c) produced by the two treatments. Subsequent treatment of PES_2 with TCA/HCl yielded only fatty aldehyde and GPE as detectable products; no lyso form could be found. This provides further evidence that PES_2 is a diplasmalogen. If PES_2 contained some 1-acyl, 2-(O-1′-alkenyl) PE, it would be unaffected by phospholipase A_2 but hydrolyzed to give the equivalent amount of lyso form by TCA/HCl, contrary to observation.

Purified PE plasmalogen from bovine brain, designated PEB, was subjected to identical experimental conditions for plasmalogen determination used with PES to check the method of analysis. Results are shown in Figure 3. PEB (Fig. 3a) subjected to TCA/HCl hydrolysis resulted in PEB_1 (15%), $LPEB_1$ (65%), fatty aldehyde (85%) (Fig. 3b, $CuSO_4$ staining) and GPE (20%) (Fig. 3c, ninhydrin staining). When PEB_1 subsequently was treated with phospholipase A_2, it was converted completely to $LPEB_2$ and fatty acid. $LPEB_1$, by contrast, was hydrolyzed completely to GPE and fatty acid when subsequently reacted with phospholipase A_2. These findings indicate that 85% of PEB contains a O-1′-alkenyl moiety at the 1 position, in agreement with the results of Touchstone et al. (18). Of that 85%, 20% is in the 1,2-di-(O-1′-alkenyl) form, since TCA/HCl treatment yielded 20% GPE (Fig. 3c), while the remaining 65% is mono(O-1′-alkenyl). On the other hand, PEB yielded PEB_2 (20%) and $LPEB_2$ (80%) after phospholipase A_2 treatment (Fig. 3d), indicating the presence of an acyl group at the 2 position in 80% of PEB molecules. The PEB fraction that was not hydrolyzed by the enzyme (PEB_2) yielded only GPE and fatty aldehyde after TCA/HCl treatment. The lyso form $LPEB_2$ yielded GPE and fatty aldehyde (65% of total PEB) and a lyso form designated $LPEB_3$ (15% of total PEB) when treated with TCA/HCl. The PEB can, from these results, be resolved tentatively into three fractions: PEB_1 (1,2 diacyl PE), 15%; PEB_2 [1,2-di(O-1′-alkenyl)], 20%, and PEB_3 [1-(O-1′-alkenyl)-2-acyl PE], 65%. The lyso PE products also can be assigned from the above distribution: $LPEB_1$, 2-acyl lysoPE; $LPEB_2$, 1-acyl lysoPE and 1-(O-1′-alkenyl) lysoPE, and $LPEB_3$, 1-acyl lysoPE.

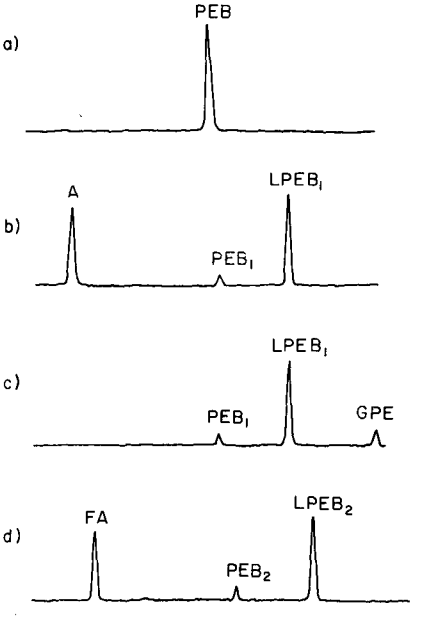


FIG. 3. Densitometric traces of TLC plates with purified phosphatidylethanolamine from bovine brain (PEB), stained with $CuSO_4$ or ninhydrin after treatment as in Fig. 2. The profile of untreated PEB is shown in trace *a*; that of PEB treated with the TCA/HCl reagent is shown in trace *b*. Staining was with reagent $CuSO_4$. The profile of PEB treated with the TCA/HCl reagent and stained with ninhydrin is shown in trace *c*; that of PEB treated with phospholipase A_2 and stained with $CuSO_4$ is shown in trace *d*.

Further support for these assignments for PES and PEB was sought through ^{1}H-NMR and infrared spectroscopy. ^{1}H-NMR data for PEB and PES, along with those for DPPE as comparison, are summarized in Table 2. While the CH_2 protons adjacent to the ester group and the vinyl protons adjacent to the ether oxygen were all present in PEB, only the latter were found in PES. In addition, the $(CH_2)_n$ protons of the alkyl chains were present in both compounds. This indicates the presence of both acyl and alkenylether moieties in PEB, while alkenylether moieties predominate in PES. Peak height values for the 1 and 2 vinyl protons of PES spectra were 1.85-fold greater than those obtained for PEB. This is in agreement with the value of 1.75 calculated from the distributions of PE forms in PEB listed above and 90% diplasmalogen PE in PES.

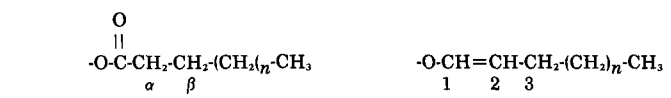
The infrared spectra of PEB, PES and DPPE are shown in Figure 4. The spectrum of DPPE showed a strong C=O absorption band at 1720

TABLE 2

Proton Nuclear Magnetic Resonance Spectra of PEB and PES, with Assignments to Functional Groups of the Phospholipids

Compound[b]	CH_3	$(CH_2)_n$	3-CH_2	β-CH_2	α-CH_2	2-CH	1-CH
II	0.88	1.31	2.02	1.57	2.26	5.35	5.87
III	0.88	1.31	2.02	N.D.[c]	N.D.[c]	5.35	5.87

[a]Positions of methylene and methine groups on acyl and alkenylether chains are defined as:



Methyl group is terminal methyl of chain.

[b]II: Phosphatidylethanolamine from bovine brain (PEB). III: Phosphatidylethanolamine from rabbit sperm (PES). Compound I, dipalmitoylphosphatidylethanolamine (synthetic DPPE) shows no detectable signals attributable to alkenylether chains, but only signals attributable to saturated alkyl chains.

[c]Not detectable.

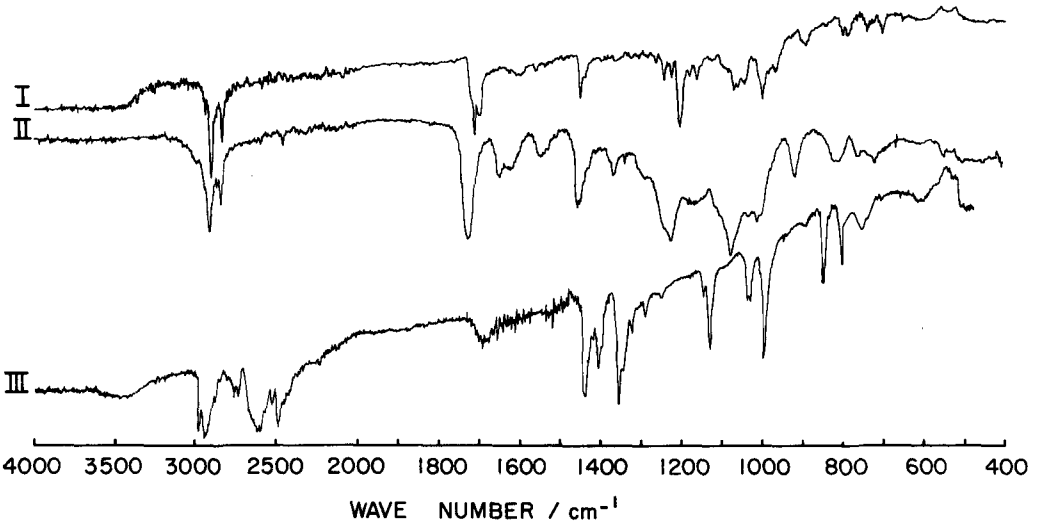


FIG. 4. Comparison of the infrared spectra of DPPE (I), PEB (II) and PES (III). Wave numbers of maximum absorbance giving absorbance bands corresponding to the functional groups in the different PE preparations are listed in Table 3.

TABLE 3

Absorption Bands and Their Assignments in the Infrared Spectra of Synthetic, Bovine Brain and Rabbit Sperm Phosphatidylethanolamines

Compound[a]	C=O	C-H	CH=CH	C-O	P=O	P-O-C	CH=CH-O
I	17.20	1450	N.D.[b]	1210	1180	1005	N.D.[b]
II	1730	1455	1330	1180	1190	995	810
III	N.D.[b]	1460	1350	1135	1190	1000	810

[a]I: Synthetic dipalmitoylphosphatidylethanolamine (DPPE); II: Phosphatidylethanolamine from bovine brain (PEB), and III: Phosphatidylethanolamine from rabbit sperm (PES).

[b]Not detectable.

cm^{-1}; moderately strong C-O stretching vibration at 1210 cm^{-1}, and moderate P=O and P-O-C absorption bands at 1180 and 1005 cm^{-1}, respectively (Table 3). The PES spectrum showed two differences when compared to the PEB spectrum: the C=O absorption band was not observed and the CH=CH-O absorption band at 810 cm^{-1} was more intense. The spectrum observed with DPPE is that expected for a diacyl phospholipid, while that observed for PEB is that expected for a monoplasmalogen. The composition of PEB (65% monoplasmalogen, 20% diplasmalogen and 15% diacylphospholipid) would give a spectrum very close to that of pure monoplasmalogen. The lack of an absorbance band for carbonyl in PES provides additional evidence for the predominance of diplasmalogen in this phospholipid.

Another sperm phospholipid partially made up of plasmalogen is PCS, as shown by the reduction in peak height in the chromatogram after TCA/HCl treatment (Fig. 1). This phospholipid was examined for plasmalogen content by the same method used for PES and PEB. PCS subjected to TCA/HCl hydrolysis resulted in PCS_1 (50%), $LPCS_1$ (50%) and fatty aldehyde. $LPCS_1$ was not hydrolyzed by phospholipase A_2. PCS exposure to the enzyme resulted in PCS_2 (50%), $LPCS_2$ (50%) and fatty acid (50%). PCS_2 was completely hydrolyzed to $LPCS_3$ and fatty aldehyde when exposed to the TCA/HCl reagent. These results indicate that PCS is 50% 1,2-diacyl PC and 50% 1-acyl, 2-(O-1′-alkenyl) PC (PCS_2). The lyso PCS generated from PCS by either TCA/HCl ($LPCS_1$) or phospholipase A_2 ($LPCS_2$) and the lyso PCS generated from PCS_2 by TCA/HCl ($LPCS_3$) all appear to be the same 1-substituted LPCS by chromatography. It should be noted that while a 1-acyl substituent is more probable, these results do not rule out a 1-alkylether substituent. Formation of the 1-substituted LPCS by rearrangement of 2-acyl LPCS by acyl migration appears unlikely, since such migrations have not been observed with this TCA/HCl reagent (18). This resolution provided a positive answer to the question: do diplasmalogens exist? It also uncovered the surprising result that 90% of rabbit sperm PE occurs as the diplasmalogen, with the remainder as diacyl PE. The sperm PC, on the other hand, has the expected composition of 50% plasmalogen as monoplasmalogen (9).

These results still leave unanswered the central biological question concerning plasmalogens: what special role do they play in biological membranes?

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Changes in Δ^5- and Δ^7-Sterols During Germination and Seedling Development of *Cucurbita maxima*[1]

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ABSTRACT

While seeds of *Cucurbita maxima* contain both Δ^5- and Δ^7-sterols, the former, which have been described earlier, now have been found to disappear during germination. This suggests that a function exists for the Δ^5-compounds only in the early part of the life cycle of *C. maxima*, unlike most of the other higher plants studied. In contrast to the Δ^5-sterols, the level of Δ^7-sterols increased during germination as well as during seedling development and maturation. The period of transition between germination and seedling development appeared to be of special importance in terms of sterol changes. This period represented a surge of sterol biosynthesis with an ontogenetic shift in sterol composition from approximately equal amounts of 24α- and 24β-ethyl stereochemistry to a predominance of the former. The sterol composition of the mature plants included only about 5% of the 24β-ethylsterols. The configurational relationships were demonstrated by high resolution ^{1}H-NMR. The sterols of the mature plants were: 25(27)-dehydrochondrillasterol, 24β-ethyl-25(27)-dehydrolathosterol, avenasterol, spinasterol, 22-dihydrospinasterol and 24ξ-methyllathosterol. Based on the changes which occurred in the relative amounts of the Δ^7-sterols, it did not appear that the Δ^5-components were being converted to their Δ^7-analogs.

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INTRODUCTION

The sterols of several genera of the family Cucurbitaceae have been studied extensively and found unusual structurally. An important peculiarity is the presence of a Δ^7-bond replacing the more commonly encountered Δ^5-unsaturation (1-6). Only a few investigators have reported the presence of Δ^5-sterols in plants of this family, and in most cases only trace amounts were found (7-9). It was, therefore, of considerable interest when we found a substantial amount (ca. 18% of the total fraction) of Δ^5-sterols distributed among a large array of seven different sterols in the seeds of *Cucurbita maxima* (10). This finding raised certain important questions. Are the Δ^5-sterols present during the plant's entire life cycle (germination, seedling development and maturation, etc.)? At what stage of development are these sterols actually synthesized, and how do they arise in the seeds? In the present investigation, we have explored the answer to the first question. The second will be addressed in a future paper.

Another peculiarity of Cucurbitaceae sterols is associated with the stereochemistry at C-24 of their side chain. It is well established that two extreme categories of plants exist in terms of the configuration at C-24 (3,11). In category I, which represents most of the examined Tracheophytes, are plants containing exclusively or primarily 24α-alkylsterols, while in category II are those containing exclusively or primarily 24β-alkylsterols (3,11). Between these extremes are the members of the family Cucurbitaceae, which have been shown to contain substantial amounts of both the 24α- and 24β-epimers (4-6,12,13). Furthermore, the relative distribution of the two epimers seems to depend on ontogeny. For example, the seeds of *Cucurbita pepo* (12), *Bryonia dioica* (5) and *Trichosanthes japonica* (6) have been shown to have large proportions of 24β-ethylsterols, whereas in the mature tissue of these plants only 24α-ethylsterols are reported to occur in consequential amounts (3,5,6). This change has been suggested as an "evolutionary recapitulation," since sterols of the great majority of the investigated nonvascular plants (algae and fungi) contain only 24β-alkylsterols (3,11,14). However, at present very little is known about the exact ontogenetic timing of such an important evolutionary event. Most investigators so far have studied the sterol patterns either in the

[1]A portion of this work was presented at the meeting of the American Oil Chemists' Society in May, 1985 in Philadelphia.

Nomenclature: Codisterol = 24β-methylcholesta-5,25(27)-dien-3β-ol; 25(27)-dehydroporiferasterol = 24β-ethylcholesta-5,22E,25(27)-trien-3β-ol; clerosterol = 24β-ethylcholesta-5,25(27)-dien-3β-ol; isofucosterol = 24Z-ethylidenecholest-5-en-3β-ol; stigmasterol = 24α-ethylcholesta-5,22E-dien-3β-ol; campesterol = 24α-methylcholest-5-en-3β-ol; sitosterol = 24α-ethylcholest-5-en-3β-ol; 25(27)-dehydrochondrillasterol = 24β-ethyl-5α-cholesta-7,22E,25(27)-trien-3β-ol; 24β-ethyl-25(27)-dehydrolathosterol = 24β-ethyl-5α-3β-ol; spinasterol = 24α-ethyl-5α-cholesta-7,22E-dien-3β-ol; 22-dihydrospinasterol = 24α-ethyl-5αcholest-7-en-3β-ol; 24ξ-methyllathosterol = 24ξ-methyl-5α-cholest-7-en-3β-ol; 25(27)-dehydrofungisterol = 24β-methyl-5α-cholesta-7,25(27)-dien-3β-ol.

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seed or in the mature plants, while the period in between largely has been ignored.

In this investigation we studied the changes which occur in the sterol profiles during germination and seedling development of *C. maxima*. Our results indicate that most of the shift in favor of the 24α-ethylstereochemistry actually occurs within a short period during the transition between germination and seedling development.

MATERIALS AND METHODS

Cucurbita maxima var. True Hubbard seeds were obtained from W. Atlee Burpee Co. (Warminster, Pennsylvania).

Seeds were germinated on moist filter paper inside petri dishes at 25 C. By visual inspection, seeds were noted to germinate uniformly, with radicle protrusion occurring between 1.5 and two days. After five days, seedlings were transferred to small glass vials containing commercial nutrient solution (10% Ortho 5-10-5 in water) and were grown in a glass house under natural light at 25 C for 10 days. The mature plants were raised in plastic pots containing potting soil at 25 C during the spring of 1984. The first harvest of seedlings was made 24 hr after the start of imbibition; this will be referred to as day 1 of the germination period, the day following as day 2, etc. The mature plants were harvested after flowering had occurred (ca. eight wk old), but before fruits were set.

The 4-desmethylsterols were extracted and separated from the neutral lipids after saponification, as previously described (13). The Δ^5- and Δ^7-mixtures were separated from each other by thin layer chromatography (TLC) (10) and then analyzed by gas liquid chromatography (GLC) and high performance liquid chromatography (HPLC). Data from the latter two methods as well as from ^{1}H-NMR in some cases were used for quantitation of the individual components. Standards either were purchased from commercial sources or were isolated from *C. maxima* seeds (10,13).

The instrumentation and techniques for the GLC and the HPLC (both analytical and preparative) have been described earlier (13). Mass spectra were obtained by direct probe (EIMS, ionizing energy 70 eV) on a Hitachi-Perkin Elmer Model RMU-6D instrument. ^{1}H-NMR spectroscopy was performed at 400 MHz at ambient temperature on a Bruker instrument, Model WH-400, in $CDCl_3$ with TMS as internal standard.

RESULTS AND DISCUSSION

Changes in Δ^5-Sterols

We have reported previously (10) that Δ^5-sterols account for ca. 18% of the total 4-desmethylsterol fraction in mature seeds of *C. maxima*. We now have found that this changes as the seeds germinate. Figure 1 shows how the percentages of Δ^5-sterols relative to the total sterols actually varied. While little or no alteration occurred during imbibition (day 1), the percentage of Δ^5-sterols sharply decreased during the 24 hr period (day 2) following the imbibition and continued to decrease during days 3 and 4 (Fig. 1). By day 5 Δ^5-sterols constituted

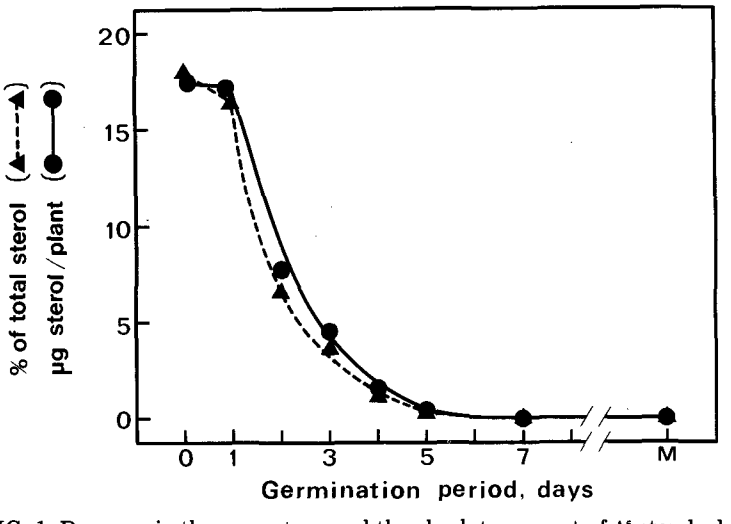

FIG. 1. Decrease in the percentage and the absolute amount of Δ^5-sterols during germination and seedling development of *C. maxima*. M = mature plant (postflowering, ca. eight wk old).

only a fraction of 1% of the total 4-desmethylsterols. In mature plants Δ^5-sterols could not be detected at all (Fig. 1) which agrees with an earlier report (15). The decrease in the percentage of Δ^5-sterols also correlated well with a decline in the absolute amount of these compounds during germination (Fig. 1). This suggests that Δ^5-sterols may be playing some functional role during the germination process, although it is not clear what such a role might be.

The origin of the Δ^5-compounds also remains to be determined. It does not appear that the Δ^5-sterols are synthesized at all in the seedlings because, as we will report in detail in another paper, label from mevalonate which appears in the Δ^7-sterols during germination and seedling development fails to become incorporated into the Δ^5-fraction. Since the Δ^5-sterols also are absent in mature plants (Fig. 1), they probably are formed in the seed itself during its development prior to germination. While sterol synthesis in developing seeds of the Cucurbitaceae family has not been studied, it has been reported in the seeds of other tracheophyte families (16–18).

Changes in the levels of individual Δ^5-sterols also were studied (Table 1). Although the amount of each component decreased on a per plant basis during germination, the rate of decline varied for different sterols. The levels of campesterol and sitosterol decreased most rapidly, and by day 5 neither of these could be detected in the seedlings (Table 1). Progressively slower rates of decline then were observed in the levels of codisterol, 25(27)-dehydroporiferasterol, stigmasterol and isofucosterol, respectively. Clerosterol disappeared at the slowest rate, and on day 5 it was the only one of the Δ^5-sterols that remained in significant amount (Table 1). These differences in disappearance rates brought about marked changes in the relative proportions of the various Δ^5-sterols. Codisterol, which accounted for ca. 35% of the total Δ^5-fraction in the seeds prior to germination, constituted only about 22% of the total fraction in 4-day-old seedlings, whereas the percentage of clerosterol increased from ca. 20% to 55% during this same period (Table 1). Similarly, the percentages of campesterol and sitosterol decreased and those of isofucosterol and stigmasterol increased during germination (Table 1). While these results might suggest temporary interconversions of the sterols prior to their complete disappearance, the data do not readily support this possibility. For instance, based on theoretical considerations, codisterol could go either to 22-dihydrobrassicasterol by reduction of the 25(27)-double bond or to 24β-methylcholesta-5,22,25(27)-trien-3β-ol by dehydrogenation; however, neither of these two sterols was detected during germination. Similarly, the well known conversion of isofucosterol to sitosterol did not appear to be occurring in the *C. maxima* seedlings, since the amount of sitosterol relative to that of isofucosterol actually decreased (Table 1).

TABLE 1

Levels of Individual Δ^5-Sterols During Germination of *C. maxima*[a]

Sterol	Germination period, days (Amount in μg/plant)					
	0[b]	1	2	3	4	5
Codisterol	6.17 (35.3)	6.07 (35.0)	1.94 (26.6)	1.02 (23.1)	0.41 (21.9)	traces —
25(27)-Dehydroporiferasterol	0.75 (4.3)	0.74 (4.3)	0.25 (3.4)	0.11 (2.5)	traces —	traces —
Clerosterol	3.45 (19.8)	3.57 (20.6)	2.38 (32.6)	1.49 (33.8)	1.02 (54.5)	0.35 —
Isofucosterol	1.30 (7.4)	1.34 (7.7)	1.01 (13.8)	0.59 (13.4)	0.19 (10.2)	traces —
Stigmasterol	1.90 (10.9)	2.11 (12.2)	0.93 (12.7)	0.76 (17.2)	0.25 (13.4)	traces —
Campesterol	0.99 (5.7)	0.91 (5.2)	0.20 (2.7)	0.07 (1.6)	traces —	N.D. —
Sitosterol	2.89 (16.6)	2.60 (15.0)	0.60 (8.2)	0.37 (8.4)	traces —	N.D. —

[a]Values in parentheses refer to the percentages of individual components in the total Δ^5-fraction. N.D. = not detected.

[b]Day 0 represents the seed just before imbibition.

Finally, the possibility of the conversion of these Δ^5-sterols to their Δ^7-analogs also can be suggested from the results discussed in the following section. Such a conversion is not possible for the major Δ^5-sterol, codisterol (35.3% of the total), since its Δ^7-analog, 25(27)-dehydrofungisterol, which was present in trace amount in the seed, also disappeared during the germination period (Table 2). The data for other sterols is less clear. Although the levels of their Δ^7-analogs increased in most cases (Table 2), it is difficult to determine whether this is due to independent synthesis of Δ^7-components, conversion from Δ^5-sterols, or a combination of both. Clearly, the precise fate of the Δ^5-sterols remains an open question. It is noteworthy, however, that such a sudden disappearance of a complete sterol fraction hitherto has not been encountered in the plant kingdom.

Changes in Δ^7-Sterols

The changes in Δ^7-sterols were studied both on a fresh weight and a per plant basis (Fig. 2). The amount of sterol slowly decreased on a fresh weight basis during germination and appeared to approach a constant value in developing seedlings (day 13). This is expected, since a large increase in fresh weight during germination occurs due to the uptake of water. Conversely, on a per plant basis the level of Δ^7-sterols rose during the entire sampling period (Fig. 2). Overall there was about a five-fold increase in their amount during the first 13 days of germination. This increase, however, did not occur at a constant rate and appeared to depend on the developmental stage of the seedlings (Fig. 2). Based on the pattern of increase, three distinct phases were evident during the sampling period. In phase I, which occurred during the initial stages of germination (the first three days), the sterol level increased at a very slow rate (Fig. 2), and most of this increase actually was accounted for by just one sterol, avenasterol (Table 2). Avenasterol is known to act as an intermediate of 24α-ethylsterols (11) and probably is acting as one in this case also, since its proportion declined during the sharp increases in the levels of 22-dihydrospinasterol and spinasterol that were observed in phase II (Table 2). Phase II occurred during the transitional period between germination and seedling development (between days 3 and 7) and was characterized by a rapid rate of increase in the level of the total Δ^7-sterol (Fig. 2). However, in this phase almost all of the increase was represented by the two 24α-ethylsterols (Table 2). The levels of other sterols increased only temporarily between days 3 and 5, and declined between days 5 and 7 (Table 2). Phase III occurred after day 7 during the seedling development (appearance of true leaves, etc.). In this phase, the sterol amount increased at a rate intermediate between phases I and II (Fig. 2); however, the trend (in terms of individual sterols) was the same as in phase II, in that all of the increase was accounted for by the two 24α-ethylsterols (Table 2). Most of the sterol changes during the sampling period were confined to the 24α-components. In fact, the combined level of the two 24α-ethylsterols followed a pattern identical to that of the total Δ^7-sterols and showed the same three phases of increase (Fig. 2). On the other hand, the total amount of the 24β-ethylsterols remained more or less unchanged, except for a small temporary increase (between days 3 and 5) during the surge of biosynthesis in phase II (Fig. 2).

Based on the morphological changes in the seedlings, the fastest rate of increase in sterol content which occurred between days 3 and 7 (phase II) coincided with the transitional period between seedling emergence and seedling development. Since this transitional period also showed rapid seedling elongation, the sharp increase in sterol content probably is associated with the increased organelle genesis and new membrane synthesis that occurs after the initial germination period. This idea is consistent with earlier observations in many other plant species (17,19).

The period between days 3 and 7 (phase II) also appeared to be important in setting the trend for an ontogenetic change in sterol composition (in terms of the stereochemistry at C-24) that occurred during the growth and development of seedlings (Fig. 3). Initially, between days 3 and 5, the absolute amounts of both the 24α- and 24β-ethylsterols began to increase (Table 2). However, more 24α-ethylsterols were being formed than their 24β-analogs, and after day 5, only the two 24α-ethylsterols (spinasterol and 22-dihydrospinasterol) showed further increases (Table 2). The levels of the 24β-ethylsterols either remained unchanged [25(27)-dehydrochondrillasterol] or declined [24β-ethyl-25(27)-dehydrolathosterol] (Table 2) between days 5 and 7, leading to a dramatic change in the proportion of 24α- to 24β-ethylsterols which was present (Fig. 3). In mature plants the two 24β-ethylsterols together accounted for only about 5% of the total sterol fraction, whereas the 24α-ethylsterols together represented as much as 80% of the total fraction (Table 3). These results clearly identify for the first time the point in the life cycle of a cucurbit (transition period between germination and seedling development) where the bio-

TABLE 2

Levels of Individual Δ^7-Sterols During Germination and Seedling Development of *C. maxima*[a]

Sterol	Germination period, days (Amount in μg/plant)							
	0[b]	1	2	3	5	7	9	13
25(27)-Dehydro-chondrillasterol	15.9 (20.0)	17.4 (20.0)	20.4 (20.0)	20.8 (18.8)	48.4 (21.6)	43.2 (15.0)	46.3 (13.9)	44.7 11.2)
24β-Ethyl-25(27)-dehydrolathosterol	16.8 (21.1)	18.1 (20.8)	19.8 (19.4)	19.7 (17.8)	24.8 (11.1)	11.6 (4.0)	6.7 (2.0)	6.4 (1.6)
Avenasterol	10.1 (12.7)	14.5 (16.6)	24.0 (23.5)	26.1 (23.6)	48.7 (21.8)	30.6 (10.6)	33.9 (10.2)	30.3 (7.6)
Spinasterol	25.4 (32.0)	24.7 (28.4)	25.2 (24.6)	30.9 (27.9)	64.2 (28.7)	119.8 (41.6)	138.0 (41.5)	182.0 (45.6)
22-Dihydrospinasterol	10.3 (13.0)	12.4 (14.2)	12.9 (12.6)	13.2 (11.9)	37.7 (16.8)	83.1 (28.8)	107.8 (32.4)	135.7 (34.0)
24ξ-Methyllathosterol	0.8 (1.0)	traces —	traces —	traces —	traces —	traces —	traces —	traces —
25(27)-Dehydrofungisterol	0.2 (0.2)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

[a]Values in parentheses refer to the percentages of individual components in the total Δ^7-fraction. N.D. = not detected. Traces = about 0.05 μg.

[b]Day 0 represents the seed just before imbibition.

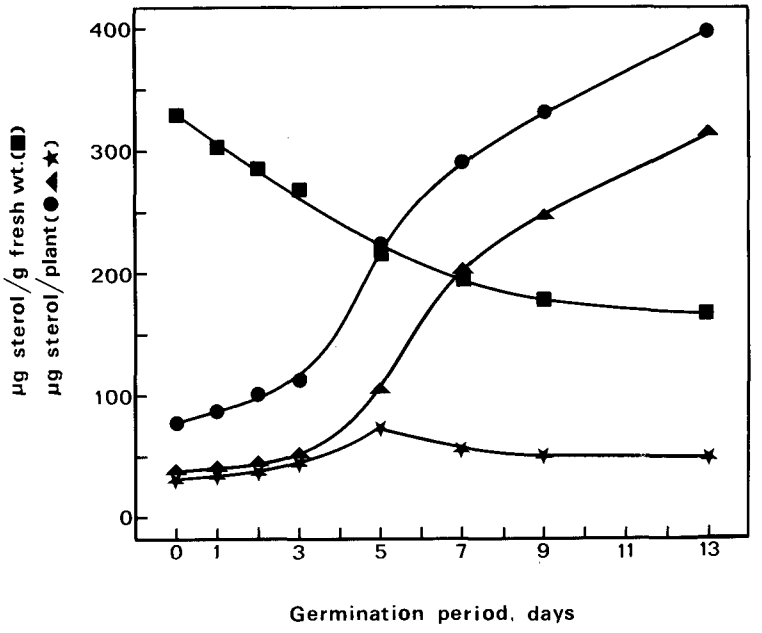

FIG. 2. Changes in the amounts of Δ^7-sterols during germination and seedling development of *C. maxima*. Total Δ^7-sterol expressed on a per g fresh wt basis (■). Total Δ^7-sterol expressed on a per plant basis (●). Total 24α- (▲) and 24β-ethyl-Δ^7-sterols (★) expressed on a per plant basis.

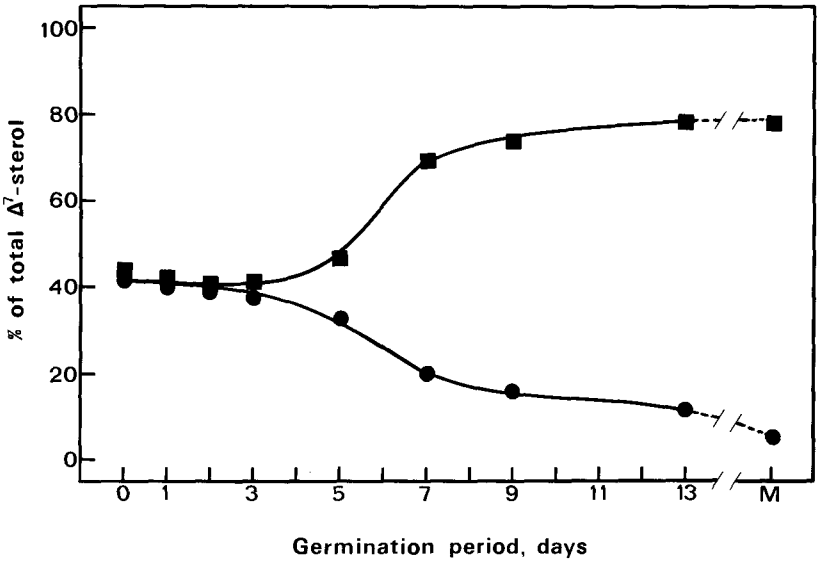

FIG. 3. Changes in the percentages of 24α- (■) and 24β-ethyl-Δ⁷-sterols (●) in the total Δ⁷-sterol during germination and seedling development of *C. maxima*. M = mature plant (post flowering, ca. eight wk old).

TABLE 3

Relative Percentages of Δ⁷-Sterols in Mature Plants[a] of *C. maxima*

Sterol	% of Total
25(27)-Dehydrochondrillasterol	1.3
24β-Ethyl-25(27)-dehydrolathosterol	3.8
Avenasterol	15.5
Spinasterol	29.6
22-Dihydrospinasterol	47.6
24ξ-Methyllathosterol	2.2

[a]Postflowering, ca. eight wk old.

synthetic shift occurs in favor of the 24α-ethylstereochemistry.

The relative distribution of individual sterols in the mature plants (postflowering, ca. eight wk old) is given in Table 3. As already indicated (Fig. 3), the mature plants contained predominantly 24α-ethylsterols; however, the proportion of 22-dihydrospinasterol relative to that of spinasterol increased during the maturation (Table 3). In contrast to the seed and seedlings (Table 2), 22-dihydrospinasterol was the most abundant sterol (47.6% of total) in mature plants (Table 3). This may represent a situation similar to the "main line" Δ⁵-series sterols in other higher plants, where the Δ⁵-monoene (sitosterol) normally is the most abundant sterol (11,17). The percentage of avenasterol in mature plants (Table 3) was about the same as in the seed (Table 2), indicating either that some independent role for this sterol exists in its own right throughout the life cycle or that the next step is regulatory, allowing this sterol to accumulate as a reserve.

Characterization of the Δ⁷-Sterols in Mature Plants

Because Δ⁵-sterols rapidly disappeared during germination, structural elucidation of the sterols in the mature plants was limited to those with a Δ⁷-bond. Special attention was given to the configuration at C-24 by the use of high resolution ^{1}H-NMR.

Four fractions (referred to as fractions 1 to 4) were separated from the mixture by preparative HPLC as described previously (13). By analytical HPLC and GLC, it was found that two of these fractions each were composed of single components. Fraction 1 showed only one peak (by analytical GLC and HPLC) with an RRt of 1.56 and an α_c of 0.71. The mass spectrum of this component gave major peaks at m/e (relative intensity): 410 (M^+, 15), 300 (10), 273 (12), 272 (26), 271 (100), 255 (24), 253 (9), 246 (10), 231 (14), 229 (12), 213 (17) and 211 (7). This fragmentation pattern was consistent with a 24ξ-ethyl-5α-cholesta-7,22,25(27)-trien-3β-ol structure (8,9,13). The final confirmation of this structure and of the configuration at C-24 was provided by ^{1}H-NMR data (Table 4). Based on considerations described earlier (13), this sterol

TABLE 4

^{1}H-NMR Chemical Shifts (δ in ppm) of Δ^7-Sterols Isolated from Mature Plants of *C. maxima*[a]

Proton	Sterol				
	25(27)-Dehydrochondrillasterol[b]	24β-Ethyl-25(27)-dehydrolathosterol	Avenasterol[f]	Spinasterol	22-Dihydrospinasterol
H-18 (3H, s)	0.545	0.526	0.538	0.551	0.536
H-19 (3H, s)	0.797	0.794	0.794	0.800	0.796
H-21 (3H, d)	1.018 (6.5)	0.910 (6.5)	0.950 (6.4)	1.026 (6.5)	0.925 (6.5)
H-26 or 27 (3H, d)	1.651[c]	1.565[c]	0.975 (6.7)	0.799 (6.1)	0.812 (6.5)
H-26 or 27 (3H, d)	4.705[d]	4.656[e] 4.734	0.975 (6.7)	0.850 (6.1)	0.835 (6.5)
H-29 (3H, t)	0.836 (7.4)	0.802 (7.2)	1.590 (6.5)[g]	0.804 (7.2)	0.846 (7.4)
H-3 (1H, m)	3.597	3.598	3.597	3.597	3.599
H-7 (1H, broad s)	5.155	5.153	5.153	5.173	5.165
H-22 or 23 (1H, dd)	5.182 (7.4)	—	—	5.027 (7.5)	—
H-22 or 23 (1H, dd)	5.260 (7.4)	—	—	5.163 (7.5)	—

[a]Values in parentheses are the coupling constants (J, in Hz). s = Singlet, d = doublet, t = triplet, m = multiplet, dd = double doublet.

[b]25(27)-Dehydrochondrillasterol also showed a peak at δ 2.421 (1H, quartet, H-24).

[c]H-26, 3H, s.

[d]H-27, 2H, s.

[e]H-27, 2H, s (terminal methylene protons resonating at two distinct δ values).

[f]Avenasterol also showed signals at δ 2.830 (1H, m, H-25) and 5.104 (1H, m, H-28).

[g]H-29, 3H, d.

was unequivocally identified as 25(27)-dehydrochondrillasterol, which has a 24β-ethyl configuration. Analytical GLC and HPLC of fraction 2 showed it to be a mixture of two sterols, tentatively identified as 24ξ-ethyl-25(27)-dehydrolathosterol (RRt 1.76, α_c 0.91) and avenasterol (RRt 1.88, α_c 0.93). As expected (13), the mass spectral analysis of both sterols produced similar fragmentation. The major peaks were m/e (relative intensity): 412 (M^+, 9), 397 (9), 315 (8), 314 (37), 299 (9), 273 (5), 272 (24), 271 (100), 255 (13), 253 (4), 246 (10), 231 (10), 229 (6) and 213 (11). This fragmentation pattern was consistent with both $\Delta^{24(28)}$- and $\Delta^{25(27)}$-structures (3,9). The structures of these two components also were established unequivocally by their ^{1}H-NMR spectra (Table 4). Signals for the $\Delta^{25(27)}$-sterol were consistent with the presence of a 24β-ethyl stereochemistry (12,13), whereas those for the $\Delta^{24(28)}$-sterol indicated a 24Z-ethylidene stereochemistry (20,21). Thus these two sterols were 24β-ethyl-25(27)-dehydrolathosterol and avenasterol.

Fraction 3 also showed the presence of two sterols: a major sterol (RRt 1.61, α_c 1.10) identified as spinasterol and a minor sterol (RRt 1.48, α_c 1.12) identified as 24ξ-methyllathosterol. The mass spectral analysis indicated that the fragmentation pattern of the major component was very similar to the literature spectrum of spinasterol (3,9,13). The major peaks were m/e (relative intensity): 412 (M^+, 38), 397 (14), 369 (18), 351 (6), 301 (7), 300 (17), 299 (10), 273 (36), 272 (28), 271 (100), 255 (50), 253 (7), 247 (10), 246 (26), 231 (18) and 213 (19). The final proof of structure for this sterol, and in particular for its C-24 configuration, again was provided by ^{1}H-NMR analysis (Table 4). Based on our earlier work (13), it is clear that this sterol is spinasterol (24α-ethyl stereochemistry) and not its 24β-epimer, chondrillasterol. The structure of the minor component could not be confirmed due to insufficient quantity. However, a mass spectral peak for the molecular ion at m/e 400 (M+), and an ^{1}H-NMR signal at δ 0.536 (3H, s) for H-18 supported the identification as 24ξ-methyllathosterol. The configuration at C-24 could not be established.

Fraction 4, which eluted last in HPLC, was shown to contain only one sterol (RRt 1.82, α_c 1.30). This was identified as 22-dihydrospinasterol. The mass spectrum of this component gave major peaks at m/e (relative intensity): 414 (M^+, 100), 399 (30), 381 (5), 273 (24), 256 (14), 255 (74), 247 (7), 246 (13), 231 (25), 229 (23) and 213 (24). This fragmentation pattern together with the ^{1}H-NMR (Table 4) confirmed the identity of the sterol as 22-dihydrospinasterol and not its 24β-epimer, 22-dihydrochondrillasterol (13). These results for the first time have confirmed the C-24 configurations of the sterols of *C. maxima* mature plants.

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Purification of the Human Anionic Polypeptide Fraction of the Apo-Bile Lipoprotein Complex by Zonal Ultracentrifugation

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ABSTRACT

The two main proteic constituents of the human Apo-bile lipoprotein complex (BLC), i.e., the anionic polypeptide fraction (APF) and the IgA fragments, were separated by preparative zonal ultracentrifugation using a sucrose gradient containing 1.5 mM glycodesoxycholate. The purification of the APF was verified by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis and immunology, and its amino acid composition then was determined. This procedure was used to obtain a polyclonal antiserum directed solely against the APF.
Lipids 20:884-889, 1985.

INTRODUCTION

It generally is admitted that the main lipid components of most mammal biles, i.e., bile salts (BS), phosphatidylcholines (PC) and free cholesterol, are transported as mixed micelles (1,2). However, results obtained in our laboratory (3-7) indicate a close association between these lipids, an APF, fragments of IgA and certain bile pigments, which together form the BLC.

According to some authors (8-13), the bile salts molecularly dispersed (as monomers or dimers) in the aqueous phase are in equilibrium with the bile salts incorporated in the lipid phase, and the physical-chemical parameters of a mixed micelle are related to the concentration of bile salts in the lipid phase. The coefficient representing the distribution of bile salts between the aqueous and lipid phases depends mostly on the hydrophobic-hydrophilic balance of their polycyclic nucleus. Using the cgs (centimeter, gram, second) system and the derivative mass/vol chemical unit mol.cm^{-3} (13), this distribution coefficient, called K_D, is equal to $BS_D/[BS_E]$, BS_D being the concentration in the micellar mixed disc and $[BS_E]$ the concentration in the aqueous phase (12,13). The radius and thus the molecular weight of a mixed micelle are inversely correlated to the BS_D concentration equal to $[BS_E]\ K_D$.

Gel filtration of mixed micelles or bile without bile salt equilibration induces a decrease of the $[BS_E]$ value due to the inclusion of bile salt monomers and dimers into the gel ($BS_D \leftrightharpoons [BS_E] \rightarrow$ inclusion). The PC and free cholesterol are eluted at the void volume, respectively, as unilamellar vesicles or as the "detergent-free form" of the BLC, corresponding to a molecular weight >2,000 kDa (3,4,12,13). The detergent-free form of the BLC is organized into lipoproteic vesicles which can encapsulate various bile proteins, such as albumin (14), IgA and IgG (7).

Gel filtration performed using 1 mM glycodesoxycholate (GDC) for equilibration makes it possible to clearly discriminate between mixed micelles and the BLC of human hepatic bile (12,13). Elution of mixed micelles results in a single lipid peak with an apparent molecular weight averaging 100 kDa, corresponding to a $[GDC_E]$ value of 10^{-6} mol.cm^{-3} (or 1 mM), while the elution of the BLC in human hepatic bile results in three families. The excluded family I consists of lipoproteic vesicles with a molecular weight >2,000 kDa. Family II consists of large discoidal lipoproteic structures with an apparent molecular weight between 500 and 700 kDa, and family III, which also contains IgA fragments and APF and is called the pseudomicellar family, consists of small discoidal lipoproteic structures with a molecular weight similar to that of mixed micelles, i.e., 100 kDa. In contrast, as a result of the concentrating activity of the gall bladder which induces an increase in the $[BS_E]$ value, human gall bladder bile shows an elution pattern in which the heavy families I and II disappear in favor of the pseudomicellar family III, often the only one remaining.

The present work was undertaken to separate the proteic components of the BLC under conditions such that only the pseudomicellar family would remain, while avoiding contamination of the BLC by bile proteins. It thus was possible to dissociate the IgA fragments from the BLC, the APF still remaining associated with bile lipids.

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MATERIALS AND METHODS

Bile samples. Human gall bladder bile was obtained by peroperative puncture of the gall bladder, stored at 4 C and used within a few days. Samples containing appreciable amounts of lysophosphatidylcholine, detected by thin layer chromatography, were systematically discarded.

Synthetic bile salt-PC mixed micelles. These micelles were prepared according to the technique of Montet and Dervichian (15) and equilibrated through 48-hr dialysis with the solution used in preparative zonal ultracentrifugation.

Preparative zonal ultracentrifugation. Zonal ultracentrifugation was performed in the Beckman ultracentrifuge L5-75B with zonal rotor Ti 15. A 1-15% linear sucrose gradient (1.004 to 1.060 g/ml) in 0.05 M Tris, pH 7.5, was used. GDC was added at a concentration of 1 or 1.5 mM. An overlay composed of 200 ml 0.05 M Tris, 1% sucrose and 1 or 1.5 mM GDC was added after the sample. Ultracentrifugation was carried out at 10 C for 48 hr at 30,000 rpm. Twenty-five fractions of 60 ml then were collected. The density of each fraction was measured through refractometry. The samples had been equilibrated previously through 48-hr dialysis using the zonal solution containing 1% sucrose and 1 or 1.5 mM glycodesoxycholate.

SDS polyacrylamide gel electrophoresis. Electrophoresis was performed in 15% polyacrylamide slab gel in the presence of 1% SDS, according to the method of Laemmli (16). The reference proteins were lysosyme (mol wt: 5.7 kDa), betaglobulin (mol wt: 11.5 kDa), trypsinogen (mol wt: 24 kDa), Kunitz inhibitor (mol wt: 6.5 kDa) and albumin (mol wt: 66 kDa).

Chemical analysis. The distribution of bile pigments was estimated by spectrophotometry at 450 nm. Bile salts were measured using the automatic enzymatic technique of Domingo et al. (17). PC were assayed by the semiautomatic technique of Amic et al. (18) after five-day dialysis of the zonal fractions using running water to eliminate the bile salts, sucrose and Tris buffer. Amino acid analysis was performed with an aliquot of the zonal PC peak sample as follows: the dialyzed zonal fractions containing PC were pooled, lyophilized and delipidated with chloroform/methanol (2:1, v/v). This pool of delipidated zonal fractions was deposited on a column of Blue Tris Acryl (IBF, Clichy, France) in order to eliminate any eventual albumin fragments. Hydrochloric acid (5.7 N) hydrolysis of dry residues was performed in vacuum-sealed tubes at 110 C for 24, 48 and 72 hr. The amino acid analyses were performed in a 119 CL Beckman amino acid analyzer connected to a Spectraphysics integrator.

Immunological investigations. Two ml of each dialyzed zonal fraction were concentrated four times using vacuum centrifugation with a Speed Vac Concentrator, Model SVC-100H. IgA were determined on anti-IgA endoplates (Kallestad, Austin, Texas). The presence of Apo-BLC was determined by double diffusion according to the method of Ouchterlony (19) using an anti-Apo-BLC serum obtained in the laboratory (6).

Preparation of a polyclonal anti-APF serum. The dialyzed fractions containing PC, obtained after zonal ultracentrifugation with the 1.5 mM GDC, were concentrated six to seven times in a dialysis bag using dry polyvinylpyrrolydone, and then two to three times using vacuum centrifugation. Three rabbits were immunized according to the method of Henry et al. (20), each animal receiving a total of 1 mg of APF. The polyclonal anti-APF serum was tested by double diffusion using bile proteins such as albumin, IgA and IgG and using the Apo-BLC obtained in the laboratory (6).

RESULTS

Figure 1A shows the zonal ultracentrifugation pattern obtained with bile salt-PC mixed micelles, consisting of a single peak with its maximum at a density of 1.020 g/ml.

Figure 1B shows the pattern obtained with human gall bladder bile in the zonal buffer containing 1 mM GDC. The pattern shows a single peak with its maximum at a density of 1.024 g/ml. The percentage weight of protein at this maximum point with respect to the PC averages 3%. IgA fragments and APF both are present in the PC peak sample.

Figure 1C shows the pattern obtained with human gall bladder bile in the zonal buffer with 1.5 mM GDC. The pattern shows a peak with its maximum at a density of 1.027 g/ml. Immunological results are shown in Fig. 1C; the APF remains associated with the peak fraction of the PC, whereas the IgA fragments are dissociated and recovered at the bottom of the sucrose gradient, together with other bile proteins and often an insoluble orange, bilirubin-like pigment. The percentage weight of APF with respect to the PC averages 2.3%. After dialysis, lyophilization and delipidation of the PC peak fraction, the APF is recovered as a dry residue. SDS polyacrylamide gel electrophoresis shows a single band containing some pigments with an apparent mol wt of 10 kDa (Fig. 2). The amino acid composition of the APF is given in Table 1, showing a preponderance of acid and neutral amino acids.

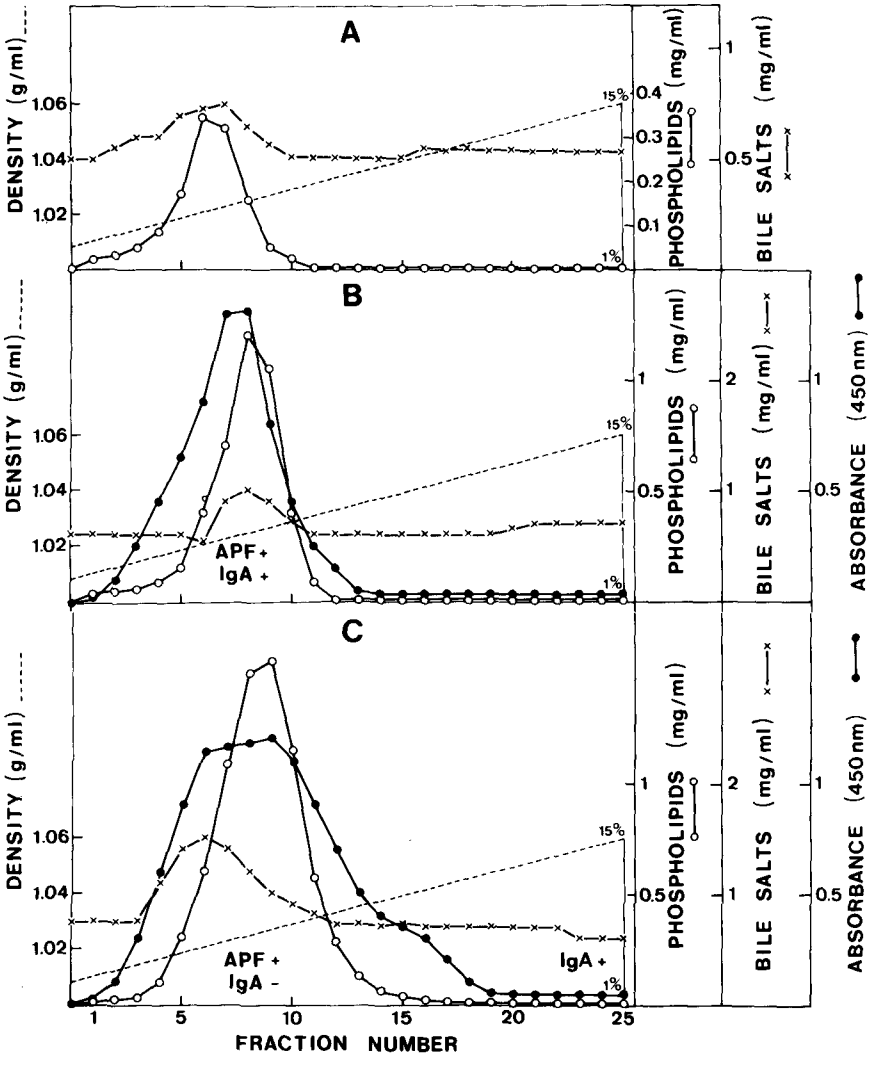

FIG. 1. Zonal ultracentrifugation patterns (see text) in 1–15% sucrose gradient. A and B: the zonal buffer contains GDC at the concentration of 1 mM. A and B, respectively BS-PC mixed micelles and human gall bladder bile, equilibrated previously through 48 hr dialysis using the zonal buffer containing 1% sucrose. The bile PC peak contains both IgA fragments and APF. C: the zonal buffer contains GDC at the concentration of 1.5 mM. Typical pattern of a human gall bladder bile equilibrated previously through 48 hr dialysis using the zonal buffer containing 1% sucrose. The PC peak only contains APF. GDC, glycodesoxycholate; BS, bile salt; PC, phosphatidylcholine.

Figure 3 shows the APF tested through double diffusion using the anti-human APF serum prepared from two rabbits. Figures 4A and 4B show that the polyclonal antibodies that react against APF do not react against the other components in bile such as IgA, IgG and albumin. Polyclonal anti-APF serum reveals a reaction identical to that obtained with one of the proteic components of Apo-BLC obtained in the laboratory, composed of APF and IgA fragments.

DISCUSSION

In models of BS-PC-free cholesterol mixed micelles proposed by Small (1), Mazer et al. (2) and Hauton et al. (12,13), two different lipid/water interfaces are present: a) the two

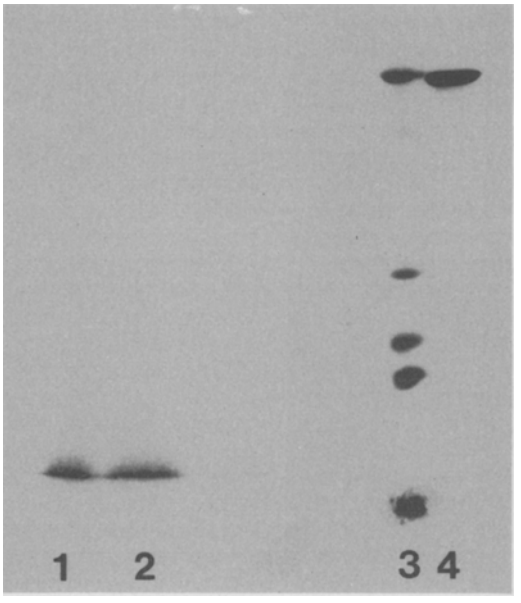

FIG. 2. Mol wt determination of APF by SDS polyacrylamide slab gel electrophoresis. Protein standards are Kunitz inhibitor (6.5 kDa), lysosome (14.3 kDa), β-globulin (18.4 kDa), trypsinogen (24 kDa) and albumin (66 kDa) treated with β-mercaptoethanol (3,4). APF (25 μg and 50 μg) purified from two different human gall bladder biles by zonal ultracentrifugation in presence of 1.5 mM GDC and treated with β-mercaptoethanol (1 and 2).

TABLE 1

Amino Acid Composition in Molar Percentage of the Human APF Purified by Zonal Ultracentrifugation in the Presence of 1.5 mM GDC[a]

Lysine	= 5.4%	Glycine	= 8.5%
Histidine	= 1.9%	Alanine	= 11.1%
Arginine	= 4.0%	Valine	= 9.8%
Asparagine	= 10.9%	Methionine	= 2.3%
Threonine	= 5.2%	Isoleucine	= 5.5%
Serine	= 4.8%	Leucine	= 10.0%
Glutamine	= 12.0%	Tyrosine	= 2.9%
Proline	= 4.1%	Phenylalanine	= 3.5%

[a]Three hydrolysis times were used (20, 48 and 72 hr). Cys and Try were not specifically determined.

discoidal surfaces of the mixed disc, called the S_D interface, consisting of a vertical packing of molecules whose polar head is directed toward the aqueous phase, and b) the annular monolayer of bile salts, called the S_A interface, in which the hydrophilic side of the bile salt polycyclic nucleus is directed toward the aqueous phase, and which may be compared to a bile salt monolayer at low surface pressure. S_D lipid/water interfaces are more favorable to electrostatic interactions than S_A lipid/water interfaces, which are favorable to hydrophobic interactions. It has been demonstrated theoretically that the preservation of a thermodynamic equilibrium requires an increase of the micellar radius if the S_D lipid/water interface is partially blocked, and a decrease of this radius if the S_A lipid/water interface is partially blocked (13). The dissociation of the IgA fragments observed during zonal ultracentrifugation in the buffer containing 1.5 mM GDC, but not in the buffer containing 1 mM GDC, suggests that these IgA fragments are associated electrostatically with S_D lipid/water interfaces, and that the electronegativity increase of such interfaces (due to the increased BS_D value induced by the increased $[BS_E]$ value) promotes this dissociation. In contrast, the fact that APF remains associated with the BLC suggests a hydrophobic interaction with S_A lipid/water interfaces. The broad peak observed after ultracentrifugation evokes a slight heterogeneity of the BLC, of which the mol wt depends on the PC/bile salt molar ratio during separation.

The APF purified through zonal ultracentrifugation produces a single band in SDS polyacrylamide gel electrophoresis and does not present any antigenic community with apo AI, apo AII, apo B, apo CI, apo CII, apo CIII, apo D, apo E and highly purified albumin, whereas Vigne et al. (6) have detected an immunological identity between the Apo-BLC and the whole serum high density lipoprotein (HDL) fraction that may be due to the presence of APF. Using radioimmunological assay procedures, Sewell et al. (21) have identified and quantified apo AI, apo AII, apo CII, apo CIII and apo B in human hepatic and gall bladder bile. Recent research using gel filtration and C_s C_l density gradient ultracentrifugation shows that bile cholesterol is secreted, at least partially, as a lipoprotein complex, independent of bile acid secretion (22). Holzbach et al. (23) also have reported the presence of proteins bound to bile lipids when using gel filtration in the presence of detergent; the electrofocalization of these proteins shows several bands with a pI between 4 and 5. Nalbone et al. (5) have shown that whole Apo-BLC produces two characteristic bands with a pI between 4.8 and 5.0, but the disadvantages of gel filtration with or without bile salt equilibration are, respectively, the coelution of bile proteins and lipids and the encapsulation of contaminating proteins in lipid vesicles. Zonal ultracentrifugation is thus a better method, since unassociated bile proteins are recovered at the bottom of the zonal rotor

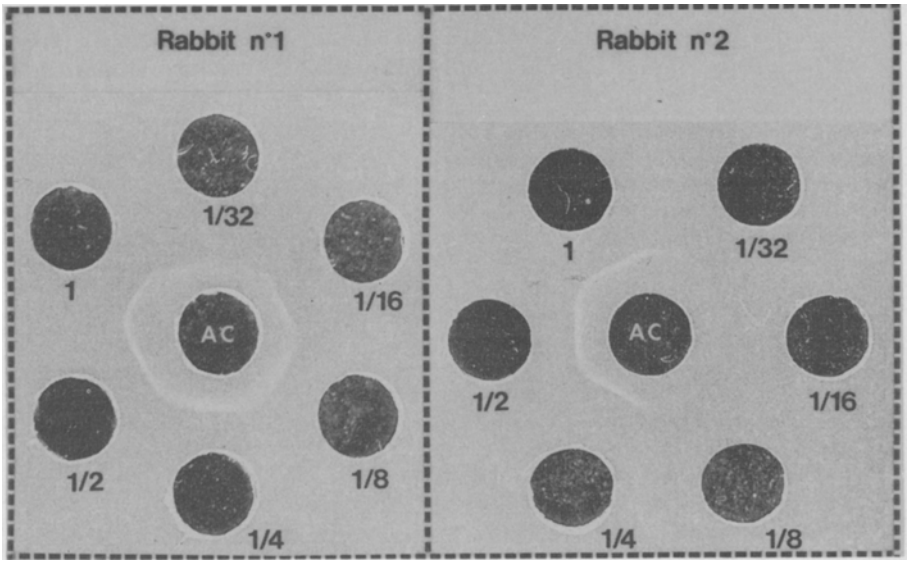

FIG. 3. Immunodiffusion plates of human APF antiserum obtained from two rabbits (center well) tested against human APF purified by zonal ultracentrifugation in presence of 1.5 mM GDC, at dilutions 1 to 1/32. The dilution one corresponds to a solubilization of 0.5 mg of APF in one ml of 0.05 M Tris, 1.5 mM GDC, pH 7.5 using ultrasonication.

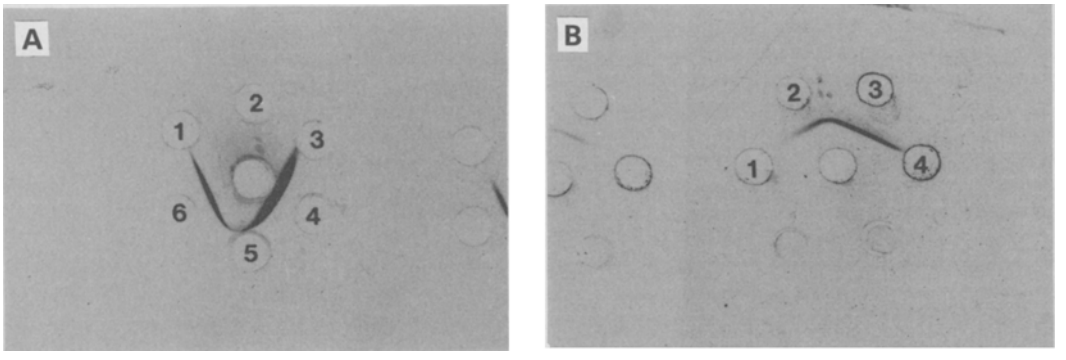

FIG. 4. A: Immunodiffusion plate of human APF antiserum (center well) tested against albumin (well 1), IgA (well 2), IgG (well 3) and APF (wells 4, 5 and 6) purified from three different human gall bladder biles by zonal ultracentrifugation in presence of 1.5 mM GDC. B: Immunodiffusion plate of human APF antiserum (center well) tested against albumin (well 1), Apo BLC (well 2), APF (well 3) and IgA (well 4). Dilution of APF corresponds to a solubilization of 0.5 mg in one ml of 0.05 M Tris, 1.5 mM GDC, pH 7.5 using ultrasonication. Other proteins, albumin, IgA and IgG, are tested in dilutions corresponding to their biliary concentrations.

where density is equal to 1.060 g/ml. Research currently underway suggests that APF is not a single polypeptide chain, but rather a polypeptidic covalent complex(es) involving covalent binding between polypeptides (20% by wt) and nonproteic components such as phosphates (2.3% by wt) and unidentified bile pigments.

Immunohistochemical studies carried out by Lafont et al. (24,25) showed that whole Apo-BLC is present both in the hepatocyte and enterocyte cells. As Apo-BLC contains both IgA fragments and APF, it was necessary to

find a method of producing specific dissociation of IgA fragments so as to obtain a preparation containing exclusively APF.

The conclusion of this study reveals APF as an apolipoproteic fraction found mainly in the hepato-biliary axis. The purification procedure described here shows that zonal ultracentrifugation can be used effectively in more extensive structural, immunological and immunohistochemical studies to provide a greater understanding of the physiological role of APF.

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Raman Studies of Structural Rearrangements Induced in Human Plasma Lipoprotein Carotenoids by Malondialdehyde

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ABSTRACT

Raman and resonance Raman spectra of plasma lipoproteins ± malondialdehyde were studied at concentrations which block the normal receptor-mediated uptake by cells. The strong resonance Raman bands at about 1010, 1162 and 1530 cm^{-1}, due to the presence of carotenoids in the lipoproteins, are envisaged as structural probes. High resolution resonance Raman spectra of the 1500–1600 cm^{-1} region reveal multiple features suggesting the coexistence of several structural populations of β-carotene whose precise assignment is complex. When plasma lipoproteins are reacted with malondialdehyde, a complex change occurs in the resonance Raman banding of β-carotene in the 1500–1600 cm^{-1} region. Malonaldehyde (MDA) also modifies the acoustical region (70–200 cm^{-1} of low density lipoprotein (LDL) lipids. We suggest that malondialdehyde association with plasma lipoproteins alters the lipid structure via apoprotein or apoprotein/lipid associations.
Lipids 20:890–896, 1985.

INTRODUCTION

It has been established that the oxidation of unsaturated acyl chains in LDL generates the crosslinker MDA as an end product, and the reaction of MDA with LDL apoprotein impairs the circulation of lipids via normal receptor-mediated endocytosis and causes LDL to be taken up more rapidly than normal by macrophages (1). We believe this results from the formation of Schiff bases with the e-amino groups of lysine residues required for the attachment of apolipoprotein B (Apo-B) to specific receptors on the surfaces of cells other than macrophages (1–4). Apart from these biological effects, the reaction of MDA with LDL changes the physical properties of the lipoprotein (5,6), leads to the cleavage of apoprotein peptide bonds (7,8) and abolishes the normal immunoreactivity (8).

It is important to obtain information about the state of carotenoids in LDL; that is, whether these molecules exist in free solution within the LDL lipid core, whether their arrangement is influenced by Apo-B, and whether their structure is altered when LDL is crosslinked by MDA, and the polyunsaturated fatty acids (9) of LDL, being subject to autocatalyzed peroxidation (10,11) and thereby acting as sources of MDA, are protected by carotenoids and other antioxidants carried by LDL.

Because LDL lipids contain carotenoids, which produce strong resonance-enhanced Raman signals, resonance Raman spectroscopy can provide information about these issues. It previously has been used to monitor temperature-induced state transitions of LDL (12); we now describe how this approach can provide some details about the arrangement of carotenoids in LDL and, by inference, about the consequences of fatty acid peroxidation and MDA-induced modifications of LDL. Our effort is facilitated by the recent normal coordinate analysis of β-carotene by Saito and Tasumi (13).

EXPERIMENTAL

Preparation of Lipoproteins

LDL and high density lipoprotein (HDL) were isolated from human plasma obtained from normal fasting donors (12 hr) as described elsewhere (14). LDL was isolated between density 1.02 and 1.063 g/cm^{-3} by repetitive ultracentrifugation at 48,000 rpm for 18 hr at 4 C. HDL was collected between densities 1.21–1.063 g/cm^{-3}.

Preparation and Assay of MDA

MDA was prepared by acid hydrolysis of malonaldehyde-*bis*-dimethylacetal (Aldrich, Milwaukee, Wisconsin). To determine the effects of MDA, we reacted 0.5 ml LDL, containing 5 mg protein per ml, with 0.25 ml 0.2M MDA at 37 C for three hr in the dark. Controls

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were treated identically but without MDA. MDA was detected by the thiobarbituric acid reaction (15).

Extraction of LDL Lipids

We isolated LDL lipids by extraction with (a) n-hexane at −10 C and (b) chloroform/methanol (2:1, v/v), as in the method of Folch et al. (16). After washing the pellets three times with their respective solvents, the supernatant fluids were mixed and evaporated, first with N_2 gas and then in vacuo. The residues then were redissolved in 1 ml of pure acetone and their resonance Raman spectra were compared with those of the pellets and untreated LDL. Protein concentration in the pellets and extracts was measured by the Lowry technique (17) and carotenoids were measured by resonance Raman spectroscopy and HPLC, using calibrated standards.

Raman Spectroscopy

Unless indicated otherwise, Raman spectra were obtained using a triple monochromator Dilor RTI/30 scanning spectrometer (Lille, France), with four slits and three 1800 g/mm plane holographic gratings. The spectrometer was interfaced to and driven by a Tracor Northern TN-1710 computer. The samples were excited using the 488 nm line of a Spectra Physics Ar^+ laser, using 20 mW for resonance Raman spectroscopy and 200 mW power in the acoustical region. For high resolution scanning, address advance = 0.1 cm^{-1}/channel was used, with slits 200–400 microns (resolution 2 cm^{-1}), scanning speed 128 cm^{-1}/min and time constant 0.5 sec.

Scanning from 800–1824 cm^{-1} was done at a faster address advance of 1 cm^{-1}/channel. Nine or more scans were accumulated in the computer memory before recording the averaged spectra on a strip chart recorder. The error in frequency measurement was no greater than 1.0 cm^{-1}. For temperature studies, sample tubes were placed in a Codberg RCN2 cryostat with the temperature adjusted by a temperature regulation unit (RC 100 for cooling and a Eurotherm 211 programmer/controller for heating. Spectra at ambient temperature (20 C or above) were recorded using rotating pyrex tubes, 5 mm internal diameter. The spectra shown are from different donors' plasmas.

Absorption Spectra

Absorption spectra were obtained with an Aminco DW2 spectrophotometer equipped to measure the spectra of crystalline suspensions, by a modification of the method of Balny et al. (18). The sample, in a cylindrical cuvette, was placed in a quartz Dewar flask and cooled with N_2 boiling off liquid N_2. Temperature regulation was done with a Barber-Colman regulator (Type 540); the temperature inside the cuvette was measured with a thermocouple connected to a digital millivoltmeter.

RESULTS

Native LDL

Pure LDL contain about 0.3 μg carotenoid/mg Apo-B, which, taking the Mr of Apo-B-100 as 250,000 and that of β-carotene as 537, corresponds to about 0.1 mol carotenoid per mol protein. Extraction with 2:1 chloroform methanol or n-hexane causes transfer of 90% of the carotenoid and 0.5% of the protein into the organic solvent phase, giving about 0.01 mol carotenoid per mol protein in the solvent residue and about 5 mol/mol in the solvent extract.

When excited at 488 nm laser frequency in a rotating cell at 20 mW power and 20 C, native LDL exhibit intense, resonance-enhanced Raman bands at 1530, 1160 and 1008 cm^{-1}, all due to lipoprotein carotenoids (Fig. 1) (12). The 1530 cm^{-1}, band is asymmetrical due to several bands and shoulders, as marked in Figure 2, but the major components of the lipoproteins, apolipoprotein, cholesteryl ester, triacylglycerol and phospholipid do not contribute significant Raman scattering at low laser power and do not interfere with the carotenoid bands under the conditions employed.

The assignment of the resonance-enhanced bands of LDL carotenoids is achieved by reference to the recent analysis of all-*trans* β-carotene (13). This molecule exhibits very intense bands at 1528, 1160 and 1008 cm^{-1}, respectively, representing in-phase C=C stretching vibrations in the central part of the chain ($^1B_u \leftarrow {}^1A^-_g$ transition), C—C stretching also from bonds in the central part, and in-phase combinations of in-plane rocking motions contributed by the CH_3 groups attached to C9 and C13. There are also a number of less prominent features that can be precisely assigned, as shall be seen.

C = C Stretching Region

The C=C stretching region of LDL at 22 C, obtained using a rotating cell, is asymmetrical due to superimposition on the principal band near 1530 cm^{-1} (1528 cm^{-1} in all-*trans* β-carotene) of shoulders near 1516, 1544 and 1553 cm^{-1} (12). At 37 C, the main peak of native LDL lies at

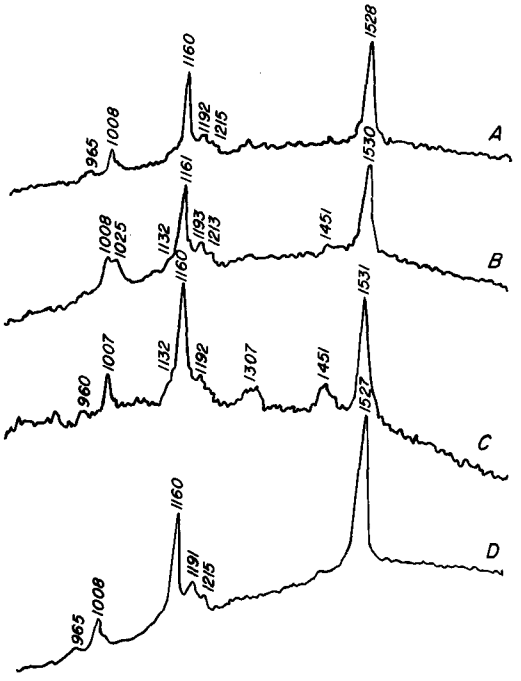

FIG. 1. Resonance Raman spectra of native LDL, supernatant and sediment after extraction with organic solvents in the 900-1600 cm^{-1} region. Excitation at 488 nm; power 20 mW; slits 400 u; address advance cm^{-1}/channel; scan speed 128 cm^{-1}/min; time constant 0.5 sec; rotating cell. Spectra are an average of 9 scans. A, Native LDL (8 mg/ml); B, sediment after chloroform/methanol extraction; C, sediment after n-hexane extraction; D, supernatant of n-hexane extract.

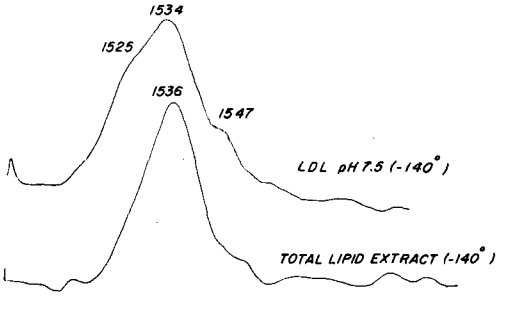

FIG. 2. Resonance Raman spectra of LDL pH 7.5 and total lipid extract at −140 C. Excitation at 488 nm; power 20 mW; address advance 1 cm^{-1}/channel; slits 400 u; scanning speed 128 cm^{-1}/min; time constant 0.5 sec. Spectra are an average of 9 scans.

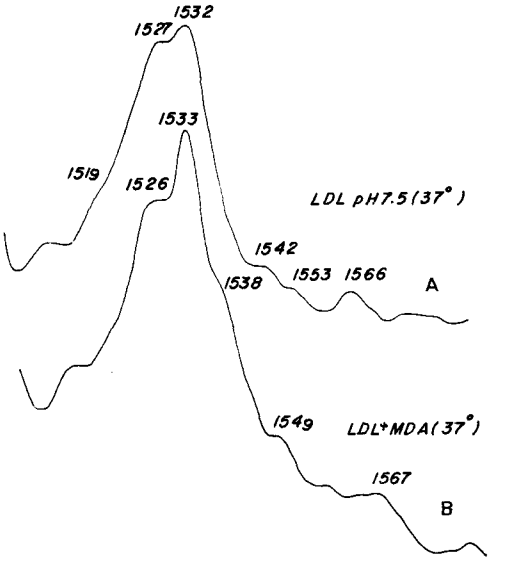

FIG. 3. Effect of MDA on resonance Raman spectra of LDL (0.3 ml of LDL [5 mg/ml; pH 7.5] incubated with 0.15 ml of MDA [0.2 mg/ml] at 37 C for 4 hr). A, Control LDL; B, LDL plus MDA. Excitation at 488 nm; power 20 mW; address advance 1 cm^{-1}/channel; slits 400 u; scanning speed 128 cm^{-1}/min; time constant 0.5 sec. Spectra are an average of 9 scans.

1532 cm^{-1}, with shoulders at 1519, 1527 and 1542 cm^{-1} (Fig. 3). At −140 C the —C=C— stretching region becomes more structured, with a shift of the main peak to 1534 cm^{-1}, heightening of the shoulder near 1525 cm^{-1} (1516 cm^{-1} at 22 C), and intensification of the shoulder at 1547 cm^{-1} (1544 cm^{-1} at 22 C) (Fig. 2). According to the analysis made by Saito and Tasumi (13), the shoulder near 1516 cm^{-1} corresponds to the strong band emitted at 1516 cm^{-1} by solid all-*trans* β-carotene, whereas the shoulders at higher frequencies, near 1544 and 1553 cm^{-1} are close in position to the features observed as well as calculated for *cis* 9 and *cis* 13 β-carotene.

The lipids extracted from LDL by chloroform/methanol (2:1, v/v) and transferred to acetone give only a single symmetrical band peaking at 1527 cm^{-1}. The full width at half height of the major C=C stretching band of LDL lipids extracted with 2:1 chloroform/methanol and transferred into 100% acetone is 12 cm^{-1}, compared with 13 and 15 cm^{-1}, respectively, for pure β-carotene in 10% and 100% acetone and native LDL (Fig. 4). The band widths are the same for monomeric and aggregated β-carotene, but the peak of the monomer lies at 1527 cm^{-1}, and of the aggregated form at 1524 cm^{-1} (12). LDL lipids extracted with n-

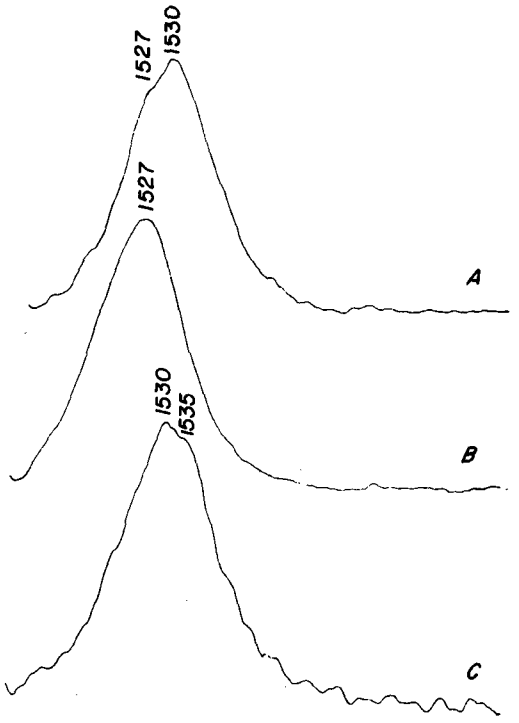

FIG. 4. Resonance Raman spectra of organic solvent extract and residue of LDL. Recording conditions as in Fig. 2. A, n-hexane extract; B, chloroform: methanol (2:1, v/v) extract; C, residue after chloroform/methanol or n-hexane extraction.

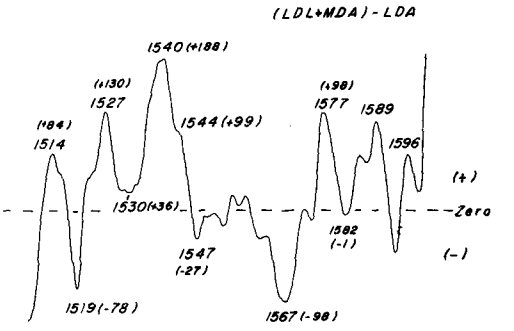

FIG. 5. Difference resonance Raman spectra ([LDL-MDA complex] − LDL) at room temperature, obtained using a rotating cell. Other recording conditions are as in Fig. 1.

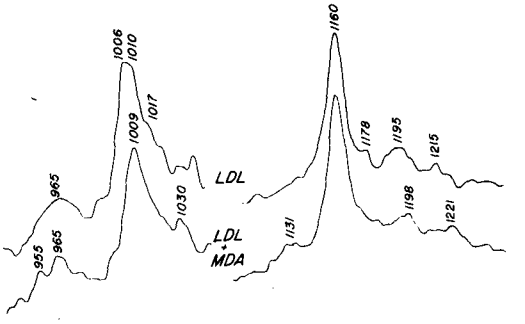

FIG. 6. Resonance Raman spectra of LDL + MDA in the 800–1050 and 1100–1300 cm^{-1} regions. Recording conditions as in Fig. 2.

hexane and transferred to acetone, in contrast, reveal a main band at 1530 cm^{-1} and a shoulder at 1527 cm^{-1} (Fig. 3A). The spectrum of the residue after n-hexane or chloroform/methanol extraction shows the main band at 1530 and shoulder at 1535 cm^{-1} (Fig. 4C).

Chloroform/methanol-extracted LDL lipids transferred to 100% acetone and measured at −140 C give a single symmetrical band at 1536 cm^{-1}, with a full width and half-height of 12 cm^{-1} (Fig. 2), close in position to that of pure β-carotene at that temperature.

Taken together, the complexity of the C=C stretching features of LDL compared with the spectral characteristics of LDL lipids in organic solution suggests that carotenoids in LDL are more constrained than they would be if dissolved in LDL lipids. Either several isomers of β-carotene coexist in LDL, or some of the β-carotene molecules are twisted or strained by interaction with other molecules in LDL.

Effect of MDA on LDL Carotenoid Signals

C=C stretching. In MDA-treated LDL, the main peaks at 1533 cm^{-1} and the shoulder at 1526 cm^{-1} become very sharp (Fig. 3), and the 1541 cm^{-1} shoulder is shifted to 1538 cm^{-1}. In addition, the difference spectrum ([MDA-modified LDL]-LDL) indicates intensification of the 1540 and 1567 cm^{-1} features (Fig. 5).

C—C stretching. The resonance Raman spectra of both native and MDA-modified LDL in the 1100–1200 cm^{-1} region (Fig. 6) are dominated by a strong 1161 cm^{-1} band representing C—C stretching of the C_{14}-C_{15} bonds in the central part of the chain (13). However, MDA-treated LDL develops a weak band at 1131 cm^{-1} not seen in native LDL, attributable to C—C stretching of the C_{10}-C_{11} bond (13). Since features mark the Raman spectra of all-*trans* solid β-carotene (1128 cm^{-1}) as well as 7 *cis*- (1134 cm^{-1}) and 9 *cis*- (1136 cm^{-1}) β-carotene molecules, but are not emitted by all-*trans* β-carotene in the cyclohexane solution, we think that the crosslinking of LDL by MDA enhances the constraint on LDL carotenoids and increases the number of β-carotene isomers that coexist within LDL.

CH_3-rocking (900–1050 cm^{-1}). In this region, the resonance Raman spectrum of native LDL is marked by a broad, medium-intensity band at 965 cm^{-1} (Fig. 6) and a strong band at 1005 cm^{-1} (perhaps mixed with a band at 1010 cm^{-1}). According to Saito and Tasumi (13), the first is attributable to CH out-of-plane wagging motions of the $C_{11}=C_{12}$ (and $C_{12'}=C_{11'}$) parts of the chain, and the second to in-phase combinations of the in-plane rocking of the CH_3 groups attached to carbons 9 and 13. Upon MDA treatment of LDL, the 965 cm^{-1} band is split into 955 cm^{-1} and 965 cm^{-1} components, of which the former can be assigned to CH out-of-plane wagging of the $C_7=C_8$ and $C_{8'}=C_{7'}$ parts of the β-carotene chain (13), and the strong 1005 cm^{-1} feature of native LDL shifts to 1009 cm^{-1}. In addition to these prominent alterations, weak bands appear at 1014, 1023 and 1034 cm^{-1}, of which the 1023 cm^{-1} and 1034 cm^{-1} bands also are observed in the 7 *cis*- and 9 *cis*-isomers of β-carotene (13).

The overall effect of MDA-crosslinking on the carotenoid resonance Raman spectra resembles the changes observed when all-*trans* β-carotene is transferred from the cyclohexane solution to the solid state (13). The solid state gives bands at 955, 965, 1008 and 1018 cm^{-1}, whereas the solution shows only two bands, at 960 cm^{-1} and 1005 cm^{-1}. The methyl-rocking data support indications given by other spectral regions that crosslinking by MDA places the carotenoid molecules into a more restrained condition.

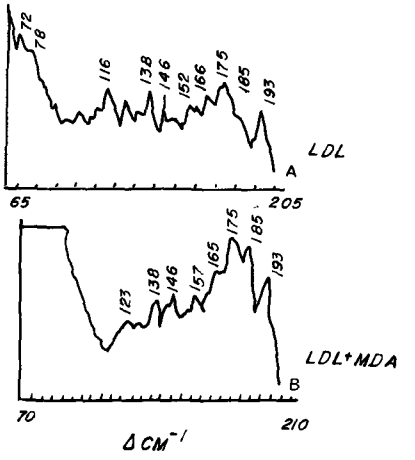

FIG. 7. Effect of MDA on the Raman spectra of LDL in the region 30–200 cm^{-1} at 20 C. A, LDL (39 mg/ml, pH 7.5); B, LDL-MDA complex. Spectra were recorded using a Codberg T800 Raman spectrometer controlled by an Apple II computer at the Laboratoire de Physique, Faculte de Pharmacie, Lille, France. Excitation at 513.5 nm; power 150 mW; slit width 2.5 cm^{-1}; address advance 0.5 cm^{-1}; channel scanning speed 128 cm^{-1}/min; time constant 0.5 sec.

Acoustical spectra. Because the LDL resonance Raman spectra do not provide direct information about LDL lipids other than carotenoids and the CH- and C—C stretching regions of acyl chains are obscured by the resonance Raman bands of carotenoids, we have examined the acoustical region 60–200 cm^{-1}, which is free of interference by resonance-enhanced carotenoid bands.

Concentrated LDL (39 mg protein/ml) gives acoustical bands at 72, 78, 116, 138, 146, 152, 166, 175 and 193 cm^{-1} and a shoulder at 185 cm^{-1} (Fig. 7). Such detail is typical of acyl chains in an ordered state, as demonstrated for cholesterol linoleate by vergoten et al. (19) and our laboratory (unpublished data). The only changes observed upon MDA treatment (Fig. 7) are the sharpening and intensification of the 185 cm^{-1} shoulder and a shift of the 116 cm^{-1} feature to 123 cm^{-1}. The results are consistent with a considerable degree of order among some LDL acyl chains and suggest that this order is increased by MDA-crosslinking.

DISCUSSION

The very strong bands at 1006, 1161 and 1530 arising from LDL are due to carotenoids (12). Although attribution of the principal peaks is straightforward, assignments for the various shoulders observed in LDL but not in organic solutions of LDL lipids can be made only according to the recent normal coordinate analysis of carotenoid resonance Raman spectra by Saito and Tasumi (13). However, even then the situation is complicated, since several minor features may arise not only from monomeric, aggregated, strained and other forms of all-*trans* carotenoids (20), but also from *cis*-isomers of β-carotenes.

The planarity of carotenoids may be distorted by interaction with proteins. Insertion of carotenoid into a hydrophobic protein pocket may occur only if the conjugated chain is strained or twisted, introducing molecular asymmetry such as might account for the induced optical activity of LDL as reported by Chen et al. (21). Induced circular dichroism studies on LDL using β-carotene as an intrinsic probe have shown that thermal transition of the lipoprotein is lowered by 1–1.5 C upon partial hydrolysis of Apo-B with trypsin (21). Similar changes have been reported by differential-scanning calorimetry (22).

Reduction of pH below 7.4 may change the orientation of LDL protein surface groups (12), and the altered LDL carotenoid resonance Raman spectra found upon lowering pH (12) may indicate a possible association of carot-

enoid with Apo-B. The same is suggested by our observation that crosslinking with MDA modifies the carotenoid resonance Raman spectra. Chio and Tappel (23) have shown that MDA reacts with the amino groups of protein lysines to form conjugated imines and also have found evidence that MDA forms intermolecular crosslinks with proteins, inactivating their biological activity in the process. It is possible, in view of these data and previously published results (5–8), that MDA-crosslinking induces a substantial reorganization of LDL, accounting for the modified carotenoid spectra we observe.

If, in crosslinking Apo-B, MDA can rigidify this molecule and thereby constrain the mobility of protein-associated lipid or carotenoid molecules, we can relate the appearance of resonance Raman spectra of MDA-treated LDL more closely to the resonance Raman bands of all-*trans* β-carotene in the solid state (13). As noted, however, no unique interpretation can be offered, since 7 *cis* and 9 *cis* β-carotene can yield overlapping features. One interpretation of the changes observed in the carotenoid spectrum following MDA-crosslinking is that a fraction of the carotenoid molecules is associated with Apo-B directly or via lipid/protein interactions. Resonance Raman analyses of β-carotene molecules intrinsic to erythrocyte ghosts (24) and platelets (25) are compatible with this concept.

Carotenoids are known to occur in association with phospholipids, regardless of whether the acyl chains are in crystalline or liquid crystalline state (26). This is shown also by resonance Raman spectra of β-carotene and egg lecithin liposomes (27), showing no shift in the frequency of carotenoid —C=C— stretching, although there is a large change of intensity as the liposomes are cycled through the crystal/liquid crystal transition. Moreover, the orientation of β-carotene molecules is sensitive to the composition of liposome acyl chains (28). The ability of β-carotene to report the thermal transition of LDL by resonance Raman spectroscopy (12) and circular dichroism (21) suggest that most of these molecules are associated with lipids in the LDL core, which structure is probably influenced by Apo-B.

The thermotropic behavior of lipid-free Apo-B has not been reported, but it has been shown that apolipoproteins influence the thermal transition of phospholipid/fatty acid liposomes (29), and our low frequency data provide proof that crosslinking of Apo-B with MDA substantially alters the LDL lipid organization (Fig. 7). It also has been shown by fluorescence spectroscopy (11) that the lipid/protein contact in oxidized LDL is less than 20% compared with 80% calculated for native LDL. Such data raise the possibility that LDL carotenoids may be associated with both Apo-B and LDL lipids.

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Influence of Hypo- and Hyperthyroidism on Rat Liver Glycerophospholipid Metabolism

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ABSTRACT

The effects of hyper- and hypothyroidism on enzyme activities involved in phospholipid metabolism in the rat liver were studied. Hyperthyroidism significantly decreases activities of both microsomal acyl-CoA:glycero-3-phosphate acyltransferase (GPAT) (34%, $p < 0.01$) and microsomal acyl-CoA:1-acylglycero-3-phosphocholine acyltransferase (GPCAT) (28–33%, $p < 0.01$). This may contribute to the decreased proportions of certain unsaturated fatty acids found in microsomal phosphoglycerides in hyperthyroidism. Mitochondrial GPAT, phospholipase A_2 and cytosol lysophospholipase are unaffected by hyperthyroidism. In contrast, hypothyroidism stimulates mitochondrial GPAT (38%, $p < 0.01$) and microsomal GPCAT (14–19%) activities but decreases both mitochondrial phospholipase A_2 (36%, $p < 0.01$) and cytosol lysophospholipase (56%, $p < 0.01$) activities. The increased GPCAT activity may contribute to the increased proportions of certain unsaturated fatty acids found in microsomal phosphoglycerides in hypothyroidism. Triiodothyronine (T_3) treatment of the hypothyroid rat (25 μg/100 g body weight/day for four days) corrected phospholipase A_2 and lysophospholipase activities to the level of the control rat, but failed to correct the increased mitochondrial GPAT activity and not only corrected but lowered GPCAT activity to the level of the hyperthyroid rat.
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INTRODUCTION

The fatty acid composition of microsomal phospholipids isolated from rat liver has been shown to be altered in diabetes (1–3), hyperthyroidism (4,5) and hypothyroidism (6,7). In diabetes and hypothyroidism, the proportions of linoleic (18:2ω6) and docosahexaenoic (22:6ω3) acids were increased, while the proportion of arachidonic (20:4ω6) acid was decreased (2,3,6). In contrast, chronic hyperthyroidism increased the proportions of stearic (18:0) and arachidonic acids and concomitantly decreased the proportions of palmitic (16:0), palmitoleic (16:1ω9), linoleic and eicosatrienoic (20:3ω6) acids (4). The increased linoleic and decreased arachidonic acid of phospholipids observed in diabetes and hypothyroidism have been thought to be due primarily to a decreased enzyme activity of Δ^6 desaturase (2,6), the rate-limiting enzyme in the conversion of linoleic acid into arachidonic acid. However, the increased 22:6ω3 levels in diabetes and hypothyroidism cannot be due to decreased desaturase activity, and may be due to diminished utilization of this fatty acid. Furthermore, Δ^6 desaturase activity also is diminished in hyperthyroidism, whereas there is an increased proportion of arachidonic acid and a decreased proportion of linoleic acid. Thus, the fatty acid composition changes in diabetes, hyperthyroidism and hypothyroidism cannot be explained by a single mechanism.

Recently, we reported that the specific activity of several enzymes involved in phospholipid synthesis and degradation in the diabetic rat were altered (8). Hypothyroidism frequently is known to coexist with diabetes (9), and decreased thyroid hormone levels occur in the experimentally diabetic animal (10). However, very little has been known about the effect of hypo- or hyperthyroidism on various metabolic enzymes involved in modifying the fatty acid composition of phospholipids. This study was carried out to determine whether hypo- or hyperthyroidism changes enzyme activities involved in the incorporation of long chain fatty acids into phospholipids; whether hypo- or hyperthyroidism has an effect on phospholipase activities involved in the degradation of phospholipids, and whether effects seen in hypothyroidism can be corrected by T_3 treatment. The enzymes studied and reported here are acyl-CoA:GPAT, acyl-CoA:GPCAT, phospholipase A_2 and lysophospholipase. GPAT is known to catalyze a rate-limiting step for the synthesis of phosphatidic acid in rat liver microsomes (11) as well as in rat liver mitochondria (12). GPCAT and phospholipase A_2 form an acylation-deacylation cycle known to play an important role in introducing polyunsaturated fatty acids (linolenic, eicosatrienoic, arachidonic) into phosphoglycerides. Finally, lysophospholipase catalyzes the degradation of phosphoglycerides. Results of this study provide further evidence that hyper- and hypo-

thyroidism as well as diabetes do affect activities of the above enzymes.

MATERIALS AND METHODS

L-*a*-glycerophosphate, fatty acid-free bovine serum albumin, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), lysophosphatidylcholine, egg lecithin, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, fatty acyl-CoAs and T_3 were obtained from Sigma Chemical Company (St. Louis, Missouri). 1-Palmitoylglycero-3-phosphate and 1-palmitoyl-*sn*-glycero-3-phosphocholine were obtained from PL Biochemicals, (Milwaukee, Wisconsin). Precoated Silica Gel G thin layer chromatography (TLC) plates were purchased from Eastman Kodak Company (Rochester, New York). Precoated Silica Gel H TLC plates were purchased from E. Merck (Darmstadt, West Germany). [U-^{14}C]-*sn*-glycero-3-phosphate was purchased from ICN Chemical and Radioisotope Division (Irvine, California). 1-Palmitoyl-2-[1′-^{14}C]-oleoyl-*sn*-glycero-3-phosphocholine, 1-[1′-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine, Aquasol-2 and Omnifluor were purchased from New England Nuclear (Boston, Massachusetts). All other chemicals and reagents were reagent grade and commercially available.

Animals and Their Treatment

Normal and thyroidectomized white male rats, strain CRl:CD(SD)BR, from Charles River Laboratories (Wilmington, Massachusetts) were maintained on a Purina Chow diet and water ad lib. At the beginning of each experiment, rats were about six wk old (aged-matched). Mean body weights were 143 g for control rats, 136 g for T_3-treated rats and 168 g for thyroidectomized rats. Hyperthyroidism was produced by daily intraperitoneal injection of 25 μg of T_3/100 g body weight for 14 days. T_3 was dissolved in 10 mM NaOH in 0.9 percent NaCl at a concentration of 0.25 mg/ml. The thyroidectomized rats were observed and weighed periodically for about three wk before use. Only those animals that demonstrated a marked decline in growth rate were considered hypothyroid. In the T_3-treated hypothyroid rats, T_3 was injected intraperitoneally once a day either at 2.0 μg/100 g body weight for seven days (low T_3 dose) or 25 μg/100 g body weight for four days (high T_3 dose). Control rats (euthyroid) were injected with equivalent amounts of 10 mM NaOH in saline (vehicle). Each experimental group consisted of four to seven rats. The number of rats killed and assayed for enzyme activities in each group are expressed in parentheses of Table 1. Rats were killed by decapitation. Blood was collected for T_3 and T_4 determinations which were done by radioimmunoassay, and the liver was removed quickly and placed in ice-cold 0.25 M sucrose. In a representative group of experimental animals, serum T_3 (ng/dl) levels (mean ± SEM) were: 101 ± 11 (control rats, n = 6), 28 ± 2 (thyroidectomized rats, n = 7) and 308 ± 38 (T_3-treated rats, n = 6). Serum T_4 (μg/dl) levels (mean ± SEM) were: 4.4 ± 0.3 (control rats, n = 6) and 0.3 ± 0.1 (thyroidectomized rats, n = 7). Mitochondria, microsomes and 100,000 g supernatants (cytosol) of the homogenized liver were isolated and kept at −70 C as described previously (8).

Enzyme Assays

Optimal conditions for each enzyme have been described previously (8). The assay procedures are as follows.

Acyl-CoA:GPAT. Reaction mixtures contained in a final volume of 0.35 ml, 60 mM Tris-HCl, pH 7.4, 0.72 mM [U-^{14}C]-*sn*-glycero-3-phosphate (specific activity 450 dpm/nmol), 2 mM $MgCl_2$, 75 μM palmitoyl-CoA and 0.5 mg mitochondrial or microsomal protein. The reaction was carried out at 37 C for three min and was terminated by adding 4 ml of chloroform/methanol (2:1, v/v) and 1 ml of 0.2 N hydrochloric acid. After centrifugation, the lower chloroform phase was washed twice with 4 ml of methanol/0.1 N hydrochloric acid (1:1, v/v) each time and counted in 10 ml of Omnifluor cocktail.

Acyl-CoA:GPCAT. This enzyme was assayed either spectrophotometrically by measuring the reduction of DTNB at 412 nm by the thiol group of free CoA-SH released or by measuring the conversion of [1-^{14}C]palmitoylglycero-3-phosphocholine to [^{14}C]phosphatidylcholine. In the spectrophotometric assay, the enzyme activity was measured at room temperature with the use of a dual beam Aminco, DW-2 UV-vis spectrophotometer set for split beam mode. The reaction mixtures consisted of 100 mM Tris-HCl, pH 7.4, 0.33 mM DTNB, 25 μM oleoyl-CoA, 0.1 mM egg lysophosphatidylcholine and 50 μg microsomal protein in a total volume of 1 ml. Egg lysophosphatidylcholine was omitted from the reference cuvette. The assay was initiated by addition of the enzyme to both reference and sample cuvettes and mixing well. The initial reaction rate was recorded continuously at 412 nm for about one min and was absolutely linear with time. In this assay procedure, because reference and sample cuvettes contained equal amounts of acyl-CoA and microsomes, microsomal acyl-CoA hydrolase activity was canceled out; thus, the changes in

TABLE 1

Effects of Hyperthyroidism and Hypothyroidism on Phospholipid Metabolism

Enzymes	Euthyroid (control)	Hyperthyroid	% Change	Hypothyroid	% Change	Hypothyroid + T_3-Treated: Low T_3 dose	% Change	Hypothyroid + T_3-Treated: High T_3 dose	% Change
GPAT (microsomal)	2.62 ± 0.12 (13)	*1.72 ± 0.04 (11)	−34	2.82 ± 0.14 (12)		2.47 ± 0.08 (5)		*1.80 ± 0.09 (7)	−31
(mitochondrial)	0.29 ± 0.02 (6)	0.28 ± 0.02 (7)		*0.40 ± 0.03 (7)	+38			*0.42 ± 0.03 (7)	+45
GPCAT									
Spectrophotometric assays	48.8 ± 1.4 (15)	*32.8 ± 1.4 (11)	−33	*58.2 ± 2.4 (19)	+19	*66.4 ± 3.3 (5)	+36	*34.6 ± 2.7 (6)	−29
Radioactive tracer assays	32.1 ± 2.1 (15)	*23.0 ± 2.5 (11)	−28	36.5 ± 1.5 (19)	+14	**40.4 ± 1.9 (5)	+26	*21.9 ± 2.2 (7)	−32
Phospholipase A_2	0.46 ± 0.03 (15)	0.44 ± 0.04 (11)		*0.29 ± 0.02 (17)	−36			0.40 ± 0.03 (7)	
Lysophospholipase	2.56 ± 0.34 (9)	2.39 ± 0.31 (7)		*1.20 ± 0.04 (12)	−56	*0.98 ± 0.08 (5)	−62	2.60 ± 0.19 (7)	

Enzyme activities were determined in triplicate. Numbers of rats in each group are shown in parentheses. Enzyme activities are expressed as nmoles/mg protein/min, except for phospholipase A_2 which is expressed as nmoles/mg protein/hr. Values are shown as mean ± SEM. Significant differences between hyperthyroid rats, hypothyroid rats and T_3-treated hypothyroid rats vs control rats are shown by *$p < 0.01$, **$p < 0.05$. Low T_3 dose = 2 μg T_3/100 g body weight/day for seven days; high T_3 dose = 25 μg T_3/100 g body weight/day for four days. Percent increase or decrease in enzyme activities with respect to control rats is shown.

optical density represented GPCAT activity. The molar extinction coefficient 13,600 M^{-1} cm^{-1} was used to calculate the enzyme activity. When the radioactive assay was used, the reaction mixture was identical with the spectrophotometric assay described above, but 0.1 mM 1-[1′-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine (specific activity 2700 dpm/nmole) was used in place of egg lysophosphatidylcholine and the incubation was carried out at 37 C for six min. The reaction was stopped by adding 4 ml chloroform/methanol (2:1, v/v) and 0.25 ml 2N sulfuric acid and mixing well. The upper aqueous phase was removed and the lower chloroform phase was washed with 4 ml chloroform/0.1 N hydrochloric acid (1:1, v/v). The washed lower phase was transferred to a new tube and small amounts of unlabeled egg lecithin and lysophosphatidylcholine carriers were added to the tube for iodine identification on TLC. After the $CHCl_3$ was evaporated using a stream of nitrogen, the residue was rinsed twice with 100 μl of chloroform, spotted on Silica Gel H and developed with chloroform/methanol/acetic acid/water (25:15:4:2, v/v) as described by Skipsky et al. (13). Lysophosphatidylcholine and phosphatidylcholine were made visible by exposure to iodine vapor. The corresponding spots were scraped and counted in 10 ml Omnifluor cocktail.

Lysophospholipase. The enzyme was assayed by determining the release of labeled fatty acid from lysophosphatidylcholine. The standard incubation mixtures contained 100 mM Tris-HCl, pH 7.4, 0.16 mM 1-[1′-^{14}C]palmitoylglycero-3-phosphocholine (specific activity 400–600 dpm/nmole) and 0.8 mg 100,000 g supernatant protein in a total volume of 1 ml. The reaction was started by adding the enzyme and incubated at 37 C for 10 min. The reaction was stopped by addition of 4 ml chloroform/methanol (2:1, v/v) and 0.25 ml 2 N sulfuric acid. After mixing to extract the [^{14}C]palmitic acid released from the reactions into the chloroform, the lower chloroform phase was washed twice with 4 ml methanol/0.1 N hydrochloric acid (1:1, v/v) each. The [^{14}C]palmitic acid was separated from unreacted substrate [^{14}C]lysophosphatidylcholine by TLC on Silica Gel G, developing with solvent system: n-hexane/diethylether/acetic acid (80:20:1, v/v). It was determined that 85–95% of [^{14}C]palmitic acid was recovered by this procedure.

Phospholipase A_2. The enzyme was assayed by the procedure described by Shakir (14) as follows: to 50 μl aliquots of 5 mM 1-palmitoyl-2-[1′-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine (specific activity 200–400 dpm/nmole) dissolved in chloroform/methanol (2:1, v/v) were added 70 μl of 0.5% Triton (v/v). The solvent then was removed at 30 C under a stream of nitrogen gas. One hundred thirty μl of buffer containing 100 mM glycine-NaOH, pH 9.5, 0.5 M KCl, 5 mM $CaCl_2$ and 2 mM sodium deoxycholate then was added. The reaction was started by adding 300 μl of rat liver mitochondria in 0.25 M sucrose containing 4 mg protein. Reaction mixtures were incubated at 37 C for three hr and reactions were terminated with 2 ml heptane/isopropanol/2 N sulfuric acid (5:20:1, v/v). The unreacted substrate and [^{14}C]oleic acid released were extracted into the heptane layer and separated by using 150 mg silicic acid as described by Shakir (14). Alternatively, to verify the amount of [^{14}C]oleic acid released, the heptane layer was evaporated, applied on Silica Gel G TLC plates and developed with solvent system n-hexane/diethylether/acetic acid (80:20:1, v/v) as described above for the enzyme lysophospholipase. These procedures gave similar amounts of [^{14}C]oleic acid released.

Statistical Analysis

The results of replicate experiments were pooled for statistical analysis; the number of animals used for each enzyme assay is indicated in Table 1. Significant differences between groups were determined by using Student's t-test.

RESULTS

Body weights of control, T_3-treated and thyroidectomized rats were determined every three days. During two wk of T_3 treatment, hyperthyroid rats increased body weight 66%, while corresponding control rats increased body weight 80%. However, during the same period, hypothyroid rats increased body weight only 14%. Retarded growth rate in addition to low serum T_3 and T_4 levels of thyroidectomized rats was taken as an indication of hypothyroidism.

Specific activities (expressed as nmoles/mg protein/min, except for the phospholipase A_2 expressed as nmoles/mg/hr) of the different enzymes which were measured in different fractions (mitochondria, microsomes or cytosol) of livers obtained from euthyroid, hyperthyroid and hypothyroid rats are expressed in Table 1.

Table 1 indicates that hyperthyroidism significantly decreased activities of both microsomal GPAT (34%, $p < 0.01$) and microsomal GPCAT. The decreased activity of GPCAT was observed with oleoyl-CoA as an acyl-CoA donor and with two independent assay procedures, the spectrophotometric assay (33%, $p < 0.01$) and the radioactive tracer assay (28%, $p < 0.01$)

that specifically measures the incorporation of [^{14}C]lysophosphatidylcholine substrate into [^{14}C]phosphatidylcholine. Hyperthyroidism, however, did not affect the enzyme activities of mitochondrial GPAT, mitochondrial phospholipase A_2 or cytosol lysophospholipase (Table 1). These data demonstrate that hyperthyroidism depresses the activity of phospholipid synthetic enzymes (GPAT and GPCAT) in microsomes but has no effect on activities of phospholipid degradative enzymes (phospholipase A_2 and lysophospholipase). This result is consistent with a recent study by Ruggiero et al. (5) who showed that hyperthyroidism decreased the phospholipid content (23%) in liver microsomes, although in an earlier study we found no change (4).

Results in Table 1 also indicate that hypothyroidism had no effect on microsomal GPAT activity, but it significantly stimulated activity of mitochondrial GPAT (38%, $p < 0.01$). The stimulation of the mitochondrial GPAT was not corrected by T_3-treatment of the hypothyroid rat. Hypothyroidism also increased the activity of microsomal GPCAT (19%, $p < 0.01$) using the spectrophotometric assay. Although the increase in GPCAT activity also was observed with the radioactive tracer assay (14%), this increase was not statistically significant. This may be because radioactive tracer assays yielded GPCAT activity 30–37% less than that measured spectrophotometrically and gave a relatively larger standard deviation than the spectrophotometric assay. Hypothyroidism also resulted in a significant decrease in both mitochondrial phospholipase A_2 (36%, $p < 0.01$) and cytosol lysophospholipase (56%, $p < 0.01$) activities. Thus, in contrast to the decreased enzyme activities of phospholipid synthesis seen in hyperthyroidism, hypothyroid rat liver increases phospholipid synthesis and simultaneously decreases enzyme activities of phospholipid degradation. Low dose T_3 treatment of hypothyroid rats did not correct for the effects of hypothyroidism on the enzymes investigated. However, high dose T_3 treatment of hypothyroid animals showed that the activities of phospholipase A_2 and lysophospholipase were corrected to the level of the control rats, but the microsomal GPAT (unaffected by hypothyroidism) and GPCAT activities were decreased to the level of the hyperthyroid rat rather than the control rat. Thus, hypothyroid rats given the larger T_3 dose not only normalized GPCAT activity to control levels, but also shifted the effects from hypothyroidism to hyperthyroidism. Thus, it appears that doses of T_3 between these two extremes or a longer treatment interval at the lower dose level will be necessary to correct GPCAT activity to the euthyroid range.

DISCUSSION

Data presented in Table 1 indicate that hyperthyroidism decreases activities of the microsomal enzymes GPAT and GPCAT involved in phospholipid synthesis in the liver. However, hyperthyroidism has no effect on activities of mitochondrial GPAT, mitochondrial phospholipase A_2 and cytosol lysophospholipase. A previous study by Roncari and Murthy (15) showed that T_4 increases triacylglycerol synthesis in the rat liver and heart. However, other investigators have found that the rate of triacylglycerol production was decreased in perfused livers from hyperthyroid rats, due to a decreased concentration of the precursor glycerol 3-phosphate (16). The increase in triacylglycerol synthesis as reported by Roncari and Murthy (15) probably is due to the increased phosphatidate phosphohydrolase (15) and diacylglycerol acyltransferase activities (17) rather than the GPAT activity, since lower incorporation of palmitate and glycerol into diacylglycerol has been described (18) consistent with the decreased GPAT activity found in the present study. The decrease of the microsomal acyltransferases GPAT and GPCAT seems consistent with the data of Ruggiero et al. (5) which showed a significant decrease (23%, $p < 0.01$) of microsomal phospholipid content in the hyperthyroid rat liver, though in an earlier study we found no change (4). Other factors such as the decreased availability of glycerol 3-phosphate (16) and diacylglycerol (18) and a greater proportion of diacylglycerol being diverted to the synthesis of triacylglycerol (17) also could contribute to the diminished synthesis of microsomal phospholipids. The effect of T_3 on the liver reported here contrasts with that found in other tissues, such as lung, in which T_3 has been shown to stimulate activities of microsomal GPCAT and phospholipase A_2 (19).

The acyltransferases GPAT and GPCAT are known to play important roles in the determination of fatty acid composition in phosphoglycerides, with the microsomal GPAT preferentially catalyzing the incorporation of saturated and monounsaturated fatty acids into phospholipids, and the microsomal GPCAT preferentially catalyzing the incorporation of polyunsaturated fatty acids into phospholipids. Therefore, the decrease in microsomal GPAT and GPCAT activities would contribute to the decreased proportions of certain unsaturated

fatty acids (palmitoleate, linoleate and eicosatrienoate) found in the microsomal phospholipids of the hyperthyroid rat liver (4). However, neither these enzyme changes nor the diminished Δ^6 desaturase activity can explain the increased arachidonate levels seen.

Our results also indicate that hypothyroidism decreases the enzyme activities of phospholipid degradation, mitochondrial phospholipase A_2 and cytosol lysophospholipase, and concomitantly increases enzyme activities of phospholipid synthesis, mitochondrial GPAT and microsomal GPCAT. The increase in GPCAT activity in hypothyroidism in contrast to the decreased activity in hyperthyroidism may explain the opposite effects of hyperthyroidism and hypothyroidism on the altered fatty acid composition of phospholipids observed in these two instances (4,6). Specifically, the increased GPCAT in hypothyroidism would increase proportions of the polyunsaturated fatty acids such as linoleate, eicosatrienoate, eicosapentaenoate and docosahexaenoate seen in microsomal phospholipids of hypothyroid rats (6). Of course, the diminished Δ^6 (6) and probable Δ^5 (20) desaturase activities in hypothyroidism would account best for the decreased arachidonate levels and would contribute to the increased linoleate and eicosatrienoate levels in hypothyroidism (6). Similar effects of streptozotocin-induced diabetes and thyroidectomy-induced hypothyroidism on the altered fatty acid composition of microsomal phospholipids in the rat liver have been shown previously (2,3,6). The results in this report also indicate that diabetes and hypothyroidism induced similar changes in the activities of several phospholipid metabolic enzymes that influence fatty acid composition in rat liver microsomes, except for mitochondrial GPAT which is increased in hypothyroidism but unchanged in diabetes (8).

The coexistence of diabetes, hypothyroidism and hyperthyroidism has been reviewed (9). Total T_4 (thyroxine), free T_4 and total T_3 levels all are significantly decreased in the streptozotocin-induced diabetic rat (10). Therefore, whether or not the effect of diabetes on phospholipid metabolism is due to the lack of insulin, the decrease of thyroid hormone or both of these hormones remains to be determined.

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Potential Antineoplastic Dihydroxy- Fatty Acids

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ABSTRACT

The fatty acid aglycones of two naturally occurring glycosides, one of which is reported to possess antitumor activity, were synthesized for biological evaluation. The preparation of 3,11-dihydroxyhexadecanoic acid and 3,12-dihydroxyhexadecanoic acid started with the monoethyl esters of nonanedioic acid and decanedioic acid, respectively, and proceeded through the corresponding C_{14} ketoesters, ketalesters, and ketalaldehydes and C_{16} ketalhydroxyesters and dihydroxyesters. Various products and intermediates were found to have no inhibitory action in the P388 lymphocytic leukemia screen. A rearrangement of the ethylene glycol ketal-protecting group from the initially protected ketone group to a newly formed aldehyde moiety was observed.
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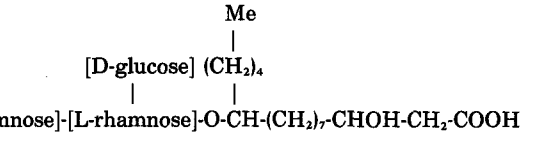
INTRODUCTION

A report by Sarin et al. (1) on the isolation and structure elucidation of ipolearoside (1), which was responsible for the significant activity of extracts of *Ipomoea leari* towards Walker carcinosarcoma 256 in rats, prompted us to prepare its aglycone, ipolearic acid (2a) or 3,11-dihydroxyhexadecanoic acid, for evaluation as an antineoplastic agent. We also chose to synthesize the aglycone of rhamnoconvolvulinic acid or 3,12-dihydroxyhexadecanoic acid (2b), whose methyl ester had been prepared before (2), to provide a similar, additional natural compound for evaluation and comparison. The route for both acids was selected to permit simple modifications for the preparation of a variety of others in which one or both hydroxyl groups could be replaced with other heteroatoms or the chain lengths between functional groups could be varied in the event the initially prepared natural acids and/or intermediates had antineoplastic activity.

RESULTS AND DISCUSSION

Syntheses were begun with the monoethyl ester of nonanedioic acid (3) for ipolearic acid and the monoethyl ester of decanedioic acid (4) for the aglycone of rhamnoconvolvulinic acid. The acid chloride of each was formed with $SOCl_2$ and used directly after verification of reaction by an infrared (IR) spectrum. The C_9-diacid derivative was added to lithium dipentylcuprate and the C_{10}-diacid derivative to lithium dibutylcuprate to form the corresponding ethyl 9-oxotetradecanoate and ethyl 10-oxotetradecanoate.

In order to provide the opportunity later to incorporate other heteroatoms at this position of the molecules and to prevent its reaction in subsequent projected steps, the ketone group of each was protected as an ethylene ketal. Both ethyl 9-ethylenedioxytetradecanoate and ethyl 10-ethylenedioxytetradecanoate formed smoothly and were identified readily from loss of IR ketone absorption at 1710 cm^{-1} and



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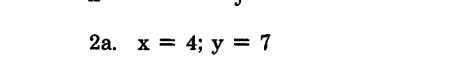

2a. x = 4; y = 7

b. x = 3; y = 8

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appearance of a nuclear magnetic resonance (NMR) singlet at δ3.8–4.0 for the four protons on the ethylene ketal.

Direct formation of a β-oxygenated acid system present at carbons 1, 2 and 3 of both target acids was attempted by reacting ethyl 10-ethylenedioxytetradecanoate with α-lithio ethyl acetate. However, no reaction ensued and only starting material was recovered. Therefore, we turned our attention to introduction of an aldehyde group at C-1, which would give the final β-hydroxyacid system directly, by reduction of the ester with diisobutylaluminum hydride (DIBAH). Initially, this reduction presented several problems: formation of copious amounts of insoluble aluminum hydroxide upon hydrolysis of the reaction; significant overreduction to the corresponding alcohol, and isolation of a reaction product containing large amounts of the acetal, 14-ethylenedioxy-6-tetradecanone, which results from the transfer of the ketal-protecting group to the newly formed aldehyde. The extent of the latter could be determined and quantified from the appearance of an NMR triplet for 1H at δ4.85 and the loss of the triplet for the aldehyde proton at δ9.7.

To counter some of these problems, the procedure was modified by employing an initial decomposition of the reaction with ethyl acetate, followed by an acetic acid-sodium acetate buffer (A. Bridges, personal communication). Various reaction conditions then were explored in order to optimize yields (Tables 1 and 2). Although the workup procedure was improved by elimination of the aluminum hydroxide and the formation of alcohols was minimized, the ratio of desired ketalaldehyde to acetalketone was not consistent. Purification of the recovered material by thin layer chromatography (TLC) gave a pure product that slowly rearranged upon storage, revealing the ketal to acetal ratio was not necessarily dependent upon reaction conditions. The aldehyde therefore was prepared and used in the next step immediately after TLC purification when gas liquid chromatography (GLC) indicated yields of greater than 60%. Reaction times of 60–90 min were favored to minimize alcohol formation.

To ascertain if the ketal exchange might be catalyzed by a factor present in the DIBAH reduction, ethyl 10-ethylenedioxytetradecanoate

TABLE 1

Reduction of Ethyl 9-Ethylenedioxytetradecanoate with DIBAH

Ester used (mmol)	DIBAH:ester (mol ratio)	Reaction (min)	Product composition (%)[a]		
			Aldehyde	Alcohol	Acetal
3.18	1	60	9.4	—	75
1.59	1	120	12	—	65.9
1.70	1.3	180	29	14	52
3.04	1.3	270	30	13.5	50
3.04	1.3	210	46	13	41
3.35	1.4	210	30	9	60
3.52	1.6	240	73.5	17.1	3.2

[a]The percent composition was determined by GLC on a 2.5% SE-30 column at 160 C.

TABLE 2

Reduction of Ethyl 10-Ethylenedioxytetradecanoate with DIBAH

Ester used (mmol)	DIBAH:ester (mol ratio)	Reaction (min)	Product composition (%)[a]		
			Aldehyde	Alcohol	Acetal
3.18	1	40	29.7	10.0	59.3
1.59	1	40	61.1	11.7	27.3
1.59	1	80	67.9	8.0	24.1
1.59	1	120	51.3	19.8	28.9
3.18	1	60	43.2	7.3	49.5
3.12	1.6	300	52.6	25	—

[a]The percent composition was determined by GLC on a 2.5% SE-30 column at 160 C.

was reduced to 10-ethylenedioxyl-1-tetradecanol, and the oxidation of this alcohol to the corresponding aldehyde was examined. The first reagent, dipyridine chromium(VI) oxide (5), produced only an acid, but lead tetraacetate in pyridine (6) gave 10-ethylenedioxytetradecanal in a 40% yield. Exchange of the protecting group still was observed with this procedure so it was not pursued, because there was no advantage in yield, product quality or ease of reaction.

The synthesis was continued with the freshly purified aldehydes by reaction with α-lithio ethyl acetate yielding ethyl 11-ethylenedioxy-3-hydroxyhexadecanoate and ethyl 12-ethylenedioxy-3-hydroxyhexadecanoate. Identification of the products came from IR bands at 3500 and 1735 for the OH and ester groups and NMR signals at δ4.2 (quartet) for the methylene portion of the ethyl ester group and at δ2.44 as a partially resolved ABX system for the methylene group α to the ester. A multiplet for the proton on the alcohol-bearing carbon also was visible at ca. δ4 along with the ethylene ketal protons. A side product resulting from addition of α-lithio ethyl acetate to the ketone of the C-1 acetal rearrangement product present in the reaction mixtures was identified from sharp IR peaks at 3520 cm^{-1} for the tertiary alcohol group and an NMR two-proton singlet at δ2.43 for the methylene protons adjacent to the ester. Both ethyl 3-acetoxy-11-ethylenedioxyhexadecanoate and ethyl 3-acetoxy-12-ethylenedioxyhexadecanoate were prepared to aid in the identification of the addition products.

In the concluding sequence of steps, the ketal groups were removed and the resultant ketohydroxyesters were reduced by $NaBH_4$ to ethyl 3,11-dihydroxyhexadecanoate and ethyl 3,12-dihydroxyhexadecanoate. Each was identified by spectral data, then hydrolyzed to ipolearic acid and the rhamnoconvolvulinic acid aglycone, respectively.

Various products and intermediates prepared were submitted to the National Cancer Institute where they were assayed in the P388 lymphocytic leukemia in vivo screening system, which has been found to be quite sensitive to antineoplastic natural products (7). At dose levels of 100, 200 and 400 mg/kg, there was no change in the life span of test animals compared to control animals. Therefore, no further compounds or derivatives were synthesized owing to the lack of activity in the models.

EXPERIMENTAL

Melting points were determined on a Fisher-Johns mp apparatus and are uncorrected. IR spectra were obtained with a Sargent-Welch 3-200 spectrophotometer on solids incorporated into KBr wafers or on neat liquids between two salt plates. NMR spectra were performed on samples dissolved in $CDCl_3$ containing tetramethylsilane as an internal reference with an IBM-NR 80 spectrometer. GLC analyses were obtained on a Varian Aerograph Series 200 f.i.d. instrument equipped with an Autolab System IV integrator.

Preparative TLC was done on 0.5 mm thick plates of silica gel PF_{254} (EM Reagents). Baker Silica Gel for Flash Chromatography was used for flash column purifications. All organic phases from extractions were dried over anhydrous $MgSO_4$.

Ethyl 9-oxotetradecanoate. A mixture of nonanedioic acid monoethyl ester (29.0 g, 0.16 mole) (3) and $SOCl_2$ (25 ml) was stirred for 24 hr; the excess $SOCl_2$ then was removed in vacuo to yield 27.5 g of acid chloride with ν_{max} 1805, 1735 cm^{-1}. The acid chloride was dissolved in ether (60 ml) and added by drops over 1 hr to dipentylcopperlithium at −78 C prepared (8) from 0.40 mole of pentyllithium (9) and 38.1 g (0.2 mole) of CuI in 450 ml ether under N_2. After 1 hr of stirring at −78 C, the reaction was quenched with absolute EtOH (10 ml), followed by dilute HCl (5%, 200 ml). The reaction was warmed to ambient temperature, the layers were separated and the aqueous portion was extracted with ether. The combined organic layers were washed with water and saturated $NaHCO_3$. The recovered product was passed through a column of flash silica gel (400 g) with 5% EtOAc/hexane to give a 78.3% yield of oxoester, bp 94–99 C (1.1 mm); ν_{max} 1730, 1710, 1180 cm^{-1}; NMR δ4.1 (q, J = 7, 2H), 2.33 (m, 6H). Anal. calcd. for $C_{16}H_{30}O_3$: C, 71.07; H, 11.18. Found: C, 70.99; H, 11.22.

Ethyl 10-oxotetradecanoate. Decanedioic acid monoethyl ester (29.3 g, 0.18 mol) (4) was converted by $SOCl_2$ (25 ml) as above to the corresponding acid chloride. The acid chloride in ether (60 ml) was reacted as above with dibutylcopperlithium (8) secured from CuI (68.1 g, 0.36 mol) in ether (800 ml) and *n*-butyllithium (447 ml, 1.6 M). Distillation of the recovered material at 0.1 mm gave a waxy solid (19.9 g, 57.8%), bp 114 C; mp 58–59 C; ν_{max} 1735, 1720, 1185 cm^{-1}; NMR δ4.12 (q, J = 7.2, 2H), 2.35 (m, 6H). Anal. calcd. for $C_{16}H_{30}O_3$: C, 71.07; H, 11.18. Found: C, 71.18; H, 11.00.

Ethyl 9-ethylenedioxytetradecanoate. A mixture of ethyl 9-oxotetradecanoate (10.2 g), ethylene glycol (39 ml), p-toluenesulfonic acid (0.4 g) and benzene (400 ml) was refluxed for 25 hr while water formed in the reaction was

collected in a Dean-Stark apparatus. The cooled solution was shaken with a saturated $NaHCO_3$ solution (600 ml), washed again with saturated $NaHCO_3$ (400 ml) and then saturated NaCl and dried. Removal of the solvent gave an oil that was passed through 300 g of flash silica gel with 10% EtOAc/hexane to yield 10.1 g (85.4%) of ketalester, bp 121-122 C (0.4 mm); ν_{max} 1732, 1180 cm^{-1}; NMR δ4.11 (q, J = 7.1, 2H), 3.98 (s, 4H), 2.28 (m, 2H). Anal. calcd. for $C_{18}H_{34}O_4$: C, 68.75; H, 10.90. Found: C, 68.87; H, 10.96.

Ethyl 10-ethylenedioxytetradecanoate. Ethyl 10-oxotetradecanoate (9.9 g) was converted to the corresponding ketalester as above to yield 9.85 g (85.6%); bp 118-119 C (0.4 mm); ν_{max} 1740, 1180 cm^{-1}; NMR δ4.11 (q, J = 7, 2H), 3.87 (s, 4H), 2.25 (m, 2H). Anal. calcd. for $C_{18}H_{34}O_6$ C, 68.75; H, 10.90. Found: C, 68.74; H, 11.17.

Aldehyde formation by DIBAH reduction. The appropriate ketalester was dissolved in dry hexane (30 ml/mmol ester) under N_2 and cooled to −78 C. DIBAH (1M hexane solution; see Tables 1 and 2 for mol equivalent used) was added over 10 min, after which the solution was stirred for the appropriate reaction time (Tables 1 and 2). Dry EtOAc (7 ml/mmol ester) was introduced over 0.5 hr to destroy excess DIBAH; stirring was continued at −78 C for 2 hr. The reaction mixture then was added to a stirred 40% HOAc solution buffered with NaOAc at 0 C. The product was recovered with EtOAc, washed with water, dried and concentrated to yield the crude aldehyde (generally equal in weight to the amount of ketalester used). The product could be purified by preparative TLC with 20% EtOAc/benzene and obtained as a colorless liquid; however, rearrangement to an acetalketone proceeded almost immediately. Spectral data are as follows:

9-ethylenedioxytetradecanal-ν_{max} 2710, 1730, 1089 cm^{-1}; NMR δ9.72 (t, 1H), 3.88 (s, 4H), 2.4 (m, 2H).

10-ethylenedioxytetradecanal-ν_{max} 2720, 1730, 1085 cm^{-1}; NMR δ9.75 (t, 1H), 3.88 (s, 4H), 2.36 (m, 2H).

14-ethylenedioxy-6-tetradecanone-ν_{max} 2940, 1735 cm^{-1}; NMR δ4.85 (t, 1H), 3.95 (s, 4H), 2.38 (m, 4H).

14-ethylenedioxy-5-tetradecanone-ν_{max} 2930, 1740 cm^{-1}; NMR δ4.85 (t, 1H), 3.95 (s, 4H), 2.38 (m, 4H).

10-Ethylenedioxy-1-tetradecanol. To a suspension of $LiAlH_4$ (1.5 g) in ether (50 ml) under N_2, a solution of ethyl 10-ethylenedioxytetradecanoate (5.87 g) in ether (10 ml) was added at a rate that maintained a gentle reflux. The reaction then was heated at reflux for 3.5 hr, decomposed at 0 C with water and acidified with 5% HCl. The product was recovered with ether and washed with water. Distillation of the residue gave the alcohol as a colorless liquid, bp 179-180 C (4.5 mm); ν_{max} 3380, 2930, 1075 cm^{-1}; NMR δ4.0 (s, 4H), 3.7 (m, 2H). Anal. calcd. for $C_{16}H_{32}O_3$: C, 70.54; H, 11.84. Found: C, 70.83; H, 12.03.

10-Ethylenedioxytetradecanal by alcohol oxidation. The prepared 10-ethylenedioxy-1-tetradecanol (0.5 g) was dissolved in dry pyridine (60 ml) containing $Pb(OAc)_4$ (0.89 g, 1 eq). The mixture was stirred at ambient temperature for 44 hr, during which time the color changed from red-brown to yellow. The pyridine was evaporated in vacuo, the residue was diluted with ether and the precipitated $Pb(OAc)_2$ was collected. The ether solution was washed twice with 10% HCl and water. Preparative TLC (20% EtOAc/hexane) gave 0.23 g (40.8%) of aldehyde identical to that prepared by DIBAH reduction.

Ethyl 11-ethylenedioxy-3-hydroxyhexadecanoate. n-Butyllithium (1.13 ml, 1.6 M in hexane) was added by drops to a stirred solution of 1,1,1,3,3,3-hexamethyldisilazane (0.4 ml, 1.87 mmole) in dry THF (5 ml) under nitrogen at −78 C. After 0.5 hr dry EtOAc (0.15 ml, 1.52 mmol) was introduced and the reaction was stirred for 10 min. Then 9-ethylenedioxytetradecanal (0.35 g, 1.3 mmol) was injected over 5 min. The reaction was quenched with a saturated NH_4Cl solution (30 ml) after 1 hr, and the product was recovered with ether. The material (0.40 g) was purified first by preparative TLC (30% EtOAc/hexane), then distilled at 123-125 C (0.2 mm) furnishing 0.19 g (42.8%); ν_{max} 3500, 1735, 1190 cm^{-1}; NMR δ4.18 (q, J = 7.1, 2H), 3.92 (s, 4H), 2.43 (ABX, J_{AX} = 7.5, J_{BX} = 5, 2H). Anal. calcd. for $C_{20}H_{38}O_5$: C, 67.00; H, 10.68. Found: C, 67.23; H, 11.01.

Ethyl 3-acetoxy-11-ethylenedioxyhexadecanoate was prepared from the product by acetic anhydride-pyridine in the usual manner. It had bp 114-115 C (0.15 mm); ν_{max} 1735, 1180 cm^{-1}; NMR δ5.25 (t, 1H), 4.13 (q, J = 7.1, 2H), 3.92 (s, 4H), 2.55 (d, J = 6.5, 2H), 2.04 (s, 3H). Anal. calcd. for $C_{22}H_{40}O_6$: C, 65.97; H, 10.07. Found: C, 65.93; H, 10.20.

A minor side product, ethyl 11-ethylenedioxy-3-hydroxy-3-pentylundecanoate, resulting from reaction with the rearranged acetalketone, also was isolated from TLC. It was identified by ν_{max} 3520, 1735, 1160 cm^{-1}; NMR δ4.8 (t, 1H), 4.13 (q, 2H), 3.85 (d, J = 3.2, 4H), 2.44 (s, 2H).

Ethyl 12-ethylenedioxy-3-hydroxyhexadecanoate. 10-Ethylenedioxytetradecanal (0.33 g) was converted to the β-hydroxyester (0.22 g, 45.7%) exactly as described above. The product had bp 104–105 C (0.15 mm); ν_{max} 3480, 1732, 1180 cm^{-1}; NMR δ4.2 (q, J = 7, 2H), 4.0 (s, 4H), 2.95 (br, 1H), 2.45 (ABX, J_{AX} = 7.5, J_{BX} = 5, 2H). Anal. calcd. for $C_{20}H_{38}O_5$: C, 67.00; H, 10.68. Found: C, 67.35; H, 10.43.

Acetylation in the usual manner gave ethyl 3-acetoxy-12-ethylenedioxyhexadecanoate, bp 107 C (0.16 mm); ν_{max} 1732, 1240, 1180 cm^{-1}; NMR δ5.23 (t, 1H), 4.15 (q, 2H), 3.94 (s, 4H), 2.55 (d, J = 6.5, 2H), 2.03 (s, 3H). Anal. calcd. for $C_{22}H_{40}O_6$: C, 65.97; H, 10.07. Found: C, 65.95; H, 10.43.

The side product ethyl 3-butyl-12-ethylenedioxy-3-hydroxydodecanoate had ν_{max} 3510, 1725, 1185 cm^{-1}; NMR δ4.85 (t, 1H), 4.18 (q, 2H), 3.89 (d, J = 2, 4H), 3.45 (br, 1H), 2.44 (s, 2H).

Ethyl 3-hydroxy-11-oxohexadecanoate. A solution of ethyl 11-ethylenedioxy-3-hydroxyhexadecanoate (0.43 g), conc. H_2SO_4 (2 drops), and water (4 ml) in acetone (60 ml) was stirred at ambient temperature for 25 hr. The acetone was removed in vacuo, the mixture was diluted with water and the product was taken up in ether. The ether layer was washed with dilute $NaHCO_3$ and water. The product was purified first on preparative TLC, then distilled to yield 0.28 g (86.8%), bp 120–122 C (0.35 mm); mp 32.5–33.5 C; ν_{max} 3450, 1730, 1710, 1185 cm^{-1}; NMR δ4.13 (q, 2H), 4.02 (br, 1H), 3.22 (m, 1H), 2.38 (m, 6H). Anal. calcd. for $C_{18}H_{34}O_4$: C, 68.75; H, 10.90. Found: C, 68.59; H, 10.59.

Ethyl 3-hydroxy-12-oxohexadecanoate. The ketal group of ethyl 12-ethylenedioxy-3-hydroxyhexadecanoate (0.7 g) was removed as above to yield 0.53 g (85.7%) of oxohydroxyester; bp 119–122 C (0.15 mm); mp 35–36 C; ν_{max} 3500, 1725, 1710 cm^{-1}; NMR δ4.18 (q, 2H), 4.0 (br, 1H), 2.87 (d, J = 3.2, 1H), 2.42 (m, 6H). Anal. calcd. for $C_{18}H_{34}O_4$: C, 68.75; H, 10.90. Found: C, 68.88; H, 10.68.

Ethyl 3,11-dihydroxyhexadecanoate. Ethyl 3-hydroxy-11-oxohexadecanoate (0.2 g) was dissolved in EtOH (50 ml) and $NaBH_4$ (0.044 g) in water (20 ml) was added. The reaction was stirred at room temperature for 24 hr; the contents then were poured into an ice and 10% HCl mixture. After 1 hr the product was recovered with ether and washed with water. The crude product was purified by preparative TLC (50% EtOAc/hexane) to yield the dihydroxyester which upon microdistillation at 120–121 C (0.3 mm) gave 0.17 g (63%); mp 52–53 C; ν_{max} 3380, 1725 cm^{-1}; NMR δ4.18 (q, J = 7.2, 2H), 4.03 (br, 1H), 3.61 (br, 1H), 2.91 (m, 1H), 2.45 (ABX, J_{AX} = 7.5, J_{BX} = 5, 2H). Anal. calcd. for $C_{18}H_{36}O_4$: C, 68.31; H, 11.47. Found: C, 67.99; H, 11.28.

Ethyl 3,12-dihydroxyhexadecanoate. Reduction of ethyl 3-hydroxy-12-oxohexadecanoate (0.27 g) with $NaBH_4$ was carried out as above. Microdistillation yielded 0.20 g (75.4%), bp 124–125 C (0.1 mm); mp 53–53.5 C; ν_{max} 3420, 1725, 1185 cm^{-1}; NMR δ4.18 (q, J = 7.2, 2H), 4.02 (br, 1H), 3.58 (br, 1H), 2.45 (ABX, J_{AX} = 7.5, J_{BX} = 5, 2H). Anal. calcd. for $C_{18}H_{36}O_4$: C, 68.31; H, 11.47. Found: C, 68.29; H, 11.18.

3,11-Dihydroxyhexadecanoic acid. A solution of ethyl 3,11-dihydroxyhexadecanoate (0.05 g) in 1% KOH/EtOH (40 ml) was stirred at room temperature for 44 hr. The reaction mixture was diluted with water and extracted with ether. The aqueous phase then was acidified with 10% HCl and the acid was recovered with ether. Recrystallization twice from MeOH/CH_2Cl_2 afforded 0.02 g (40%); mp 82.5–83.5 C (reported [1] mp 82–83 C); ν_{max} 3380, 3500–2500 (br), 1705 cm^{-1}; NMR δ4.02 (br, 1H), 3.6 (br, 1H) 2.53 (m, 2H). Anal. calcd. for $C_{16}H_{32}O_4$: C, 66.61; H, 11.18. Found: C, 66.49; H, 11.31.

3,12-Dihydroxyhexadecanoic acid. Ethyl 3,12-dihydroxyhexadecanoate (0.12 g) was hydrolyzed as above to give 0.05 g (45.7%, from MeOH/CH_2Cl_2); mp 85–86 C (reported [10] mp 84 C); ν_{max} 3420, 3500–2500 (br), 1715 cm^{-1}; NMR δ4.03 (br, 1H), 3.6 (br, 1H), 2.55 (m, 2H). Anal. calcd. for $C_{16}H_{32}O_4$: C, 66.61; H, 11.18. Found: C, 66.56; H, 11.47.

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Positional Distribution of Fatty Acids in Cardiolipin of Mitochondria from 21-Day-Old Rats

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ABSTRACT

Pure cardiolipins (1,3-diphosphatidylglycerol) were prepared from mitochondria of heart, liver and kidney from 21-day-old male Wistar rats and submitted to *Naja naja* venom phospholipase A_2 (EC 3.1.1.4) action. Incubation conditions were controlled carefully, and a complete hydrolysis of cardiolipin to lysocardiolipin {di [1 (1″) acyl *sn*-glycero-3-phosphoryl] 1′,3′-*sn*-glycerol} and fatty acids from positions 2 (2″) was obtained in less than two hr practically without side reactions. Cardiolipins from the three organs contained low levels of saturated fatty acids; stearic acid accounted for 0.4–0.7% and palmitic acid for 1.4–3.5% of total fatty acids. These percentages apparently depended on the organ. In all three cases, linoleic acid was the major component, but its percentage varied from 62–78% of total fatty acids. Acyl chains linked to positions 1 (1″) of all three cardiolipin preparations exhibited a similar pattern: they were composed of linoleic acid for 85–89%. This fatty acid also was the main component esterified at position 2 (2″), but its percentage was much more variable: from 39.8% in heart to 51.2% in kidney and 67.8% in liver mitochondria. The remaining acids comprised octadecenoic and polyunsaturated fatty acids with more than 18 carbon atoms in different proportions. As opposed to other phospholipids, *cis*-vaccenic acid, and not oleic acid, was the main octadecenoic acid present in cardiolipins.

Octadecenoic acids were nine- to 10-fold more concentrated at positions 2 (2″) than at positions 1 (1″). The percentage of *cis*-vaccenic acid was four- to five-fold higher than that of oleic acid at positions 2 (2″), whereas oleic acid dominated at positions 1 (1″). From results presented in this study and selected literature data, it may be concluded that fatty acids are asymmetrically distributed in cardiolipins of different origins, with linoleic acid showing a definite preference for position 1 (1″).

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INTRODUCTION

Selective hydrolysis of ester bonds on position 2 of phospholipids by phospholipase A_2 (phosphatide acyl-hydrolase, EC 3.1.1.4) has been described in a number of systems (1), and the location of fatty acids in phospholipids such as glycerylphosphorylcholine or ethanolamine is well documented (2). With very few exceptions (3), phospholipids from normal animal tissues contain mainly saturated and monounsaturated fatty acids at position 1 of *sn*-glycerol and almost exclusively mono- and polyunsaturated fatty acids at position 2 (2).

Cardiolipins are also substrate of phospholipase A_2 (4,5), but studies concerning the distribution of fatty acids between positions 1 (1″) and 2 (2″) are relatively scarce and discordant (6–14), and only a few of them were performed with cardiolipins of rat tissue (6–10). At least two problems seem to have hampered such studies. First, cardiolipin purification has not always been completely achieved. In many cases, fatty acid compositions with relatively high levels of saturated fatty acids have been attributed unduly to pure cardiolipins and were in fact indicative of contamination by other phospholipids (15). Second, cardiolipin hydrolysis by phospholipase A_2 proceeded very slowly in most current systems (4–9,11–14) and was not always conduced to completion (14). Incubation periods of eight to 24 hr generally were required (6–14) for this purpose and lecithins sometimes had to be added to the incubation medium, precluding analysis of fatty acids released from positions 2 (2″) of cardiolipins (11,14). Because side reactions (e.g., autoxidation, chemical hydrolysis) can occur under such lengthy conditions (14), published results are not unequivocal and some uncertainty exists as to the positional distribution of fatty acids in cardiolipin molecules. Some authors have concluded an almost random distribution of linoleic acid between positions 1 (1″) and 2 (2″) (6,8,9, 11,14) but others have observed a selective enrichment of linoleic acid at the 1 (1″)-positions (7,10,12,13).

Accumulating evidence indicates that the fatty acid profile of cardiolipin can be influenced by a number of causes (16): age (17), alcohol ingestion (18,19), hyperthyroidism (20) and dietary oils (21–25). These factors also can be responsible for alterations in fatty acid compositions of other phosphoglycerides (17–25).

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But for these lipids, modifications in the levels of saturated fatty acids occur mainly at position 1, whereas changes at position 2 essentially are related to unsaturated fatty acids. Since most cardiolipins of normal animal tissues contain only low amounts of saturated fatty acids, these particular phospholipids cannot behave in a similar way. Thus, it was of interest to gain some insight into the fatty acid distribution in cardiolipin molecules.

Because rats often are taken at weaning for dietary experiments, pure cardiolipins from heart, liver and kidney mitochondria of 21-day-old rats were prepared and submitted to the action of *Naja naja* venom phospholipase A_2 under carefully controlled conditions (26). These conditions led to a complete hydrolysis of cardiolipins in less than two hr with virtually no side reactions (26).

MATERIALS AND METHODS

Preparation of Mitochondria

Twenty male Wistar rats (mean weight 40 ± 4 g; 21 days old) were taken at weaning and decapitated. Their heart, liver and kidneys were excised rapidly and washed with ice-cold buffer (10 mM Tris, 0.25 M sucrose, 2 mM EDTA, pH 7.2). The organs were cut into small pieces with scissors, rinsed with the buffer and homogenized in the same buffer with a Potter homogenizer. For hearts only, type VIII protease from *Bacillus subtilis* (Sigma, St. Louis, Missouri) was added to the homogenate (0.8 mg protease/g of heart tissue); the suspension was stirred for 10 min and homogenized a second time. Mitochondria were prepared by successive centrifugations (27) and finally resuspended in a buffer containing 0.25 M sucrose and 10 mM Tris (pH 7.2). All operations were conducted at 4 C.

Extraction of Lipids

Mitochondrial lipids were extracted immediately and washed according to the method of Folch et al. (28), but with methanol and chloroform added successively to the mitochondrial suspensions (10). The final solvent mixture contained 0.02% (w/v) of 2,6-di-tert-butyl-4-methylphenol (BHT) as an antioxidant.

Cardiolipin Purification

Cardiolipin was separated from other lipids by preparative thin layer chromatography (TLC) on 0.3 mm thick Silica gel H (Merck, Darmstadt, West Germany) coated plates. The plates were developed with diethyl ether/acetone (90:30, v/v) and, after evaporation of the solvents, with chloroform/methanol/water (65:25:4, v/v/v) in the same direction. The second migration was stopped when the solvent front reached a line 3 cm beneath the first solvent front. The two solvent mixtures contained 0.02% BHT (w/v). The plates were sprayed with a 0.2% solution of 2′,7′-dichlorofluorescein (DCF) in ethanol and phospholipid classes visualized under UV light. Bands with the same relative mobility as authentic commercial beef heart cardiolipin (Sigma) were localized and the gel immediately scraped and transferred into test tubes. The gel was wetted with distilled water and cardiolipin was extracted three times with chloroform/methanol (2:1, v/v) and once with pure methanol. This extraction step was shown by phosphorus assay to be quantitative. Pooled extracts were taken to dryness under a stream of nitrogen at 30 C and cardiolipin was dissolved in chloroform/methanol (2:1, v/v). This solution was washed once with 0.2 vol of a 0.8% KCl aqueous solution and several times with portions of Folch upper phase (28) to remove most of the DCF. Although this washing procedure caused a small loss of cardiolipin, this did not affect the fatty acid composition of cardiolipin, which remained identical to the composition determined before its extraction from the gel (26). Solvents were evaporated under a stream of nitrogen; cardiolipin was dissolved in chloroform/methanol (2:1, v/v) and stored at −20 C under nitrogen if not used immediately. Storage never exceeded one night before use for phospholipase A_2 hydrolysis.

Hydrolysis of Cardiolipin with Phospholipase A_2

Hydrolysis of cardiolipin with phospholipase A_2 was performed according to a method already described (26). Aliquots of the cardiolipin solutions were taken to dryness and the phospholipid dissolved in diethyl ether containing 0.02% BHT (w/v). The concentration of cardiolipin was about 150 nmol/ml. A solution of phospholipase A_2 was prepared daily by dissolving 2.5 mg of a lyophilized preparation of phospholipase A_2 from *Naja naja* (290 units/mg solid, Sigma) in 0.5 ml of a 3 mM $CaCl_2$ solution. To 1 vol of the cardiolipin solution was added 0.1 vol of enzyme solution, and incubations were conducted under magnetic stirring in a water bath at 30 C for two hr. The reaction was stopped by evaporation of diethyl ether and addition of chloroform/methanol (2:1, v/v).

Hydrolysis products were separated by TLC on Silica gel H coated plates that had been prerun with diethyl ether/acetone (90:30, v/v).

The plates were developed with hexane/diethyl ether/acetic acid (90:10:1, v/v/v) to isolate the free fatty acids and with diethyl ether/acetone (60:20, v/v) followed by chloroform/methanol/water (65:25:4, v/v/v) in the same direction to separate the other hydrolysis products. BHT was added to these solvents (0.02%, w/v) when fatty acid methyl esters had to be prepared. After development, bands were revealed with the DCF solution, and the gel was scraped off and transferred to Teflon-lined screw-capped tubes for methyl ester preparation.

Methyl Ester Preparation and Analysis

Methyl esters were prepared according to Morrison and Smith (29). A methanolic solution (0.5 to 0.8 ml) of boron trifluoride (10%, w/v; Fluka, Hauppauge, New York) was added to the gel and methylation was performed at 90 C for 20 min.

Fatty acid methyl esters were analyzed by gas liquid chromatography (GLC) with a Carlo Erba HRGC apparatus equipped with a flame ionization detector. Methyl esters were separated in a glass capillary column (50 m × 0.3 mm I.D.) coated with Carbowax 20M and programmed from 65 C to 185 C (10 C/min) or operated isothermally at 185 C. Peaks were identified by comparison of relative retention times to those of commercially available methyl ester standards. Peak identification was supported by GLC analysis of methyl esters after fractionation by TLC on layers of Silica gel H containing 5% silver nitrate (by weight). These plates were developed twice with hexane/diethyl ether/acetic acid (94:4:2, v/v/v) (30).

Peak areas and percentages were calculated by a Shimadzu ICR 1A integrator.

RESULTS AND DISCUSSION

Fatty Acid Compositions

Cardiolipins freshly isolated by preparative TLC gave a single spot when rechromatographed (26). It has been shown that the chromatographic behavior of cardiolipin was influenced by metal ions present in different silica adsorbents (31). Thus, mitochondria cardiolipin might have been split in monovalent and divalent salt forms during preparative TLC (31) and resolved in two bands, and some cardiolipin could have been lost in another phospholipid fraction. This possibility was ruled out by rechromatography of all three phospholipids (glyceryl phosphorylethanolamine, -choline and -inositol) eluted from bands migrating behind cardiolipin after preparative TLC. If monovalent salts of cardiolipin were mixed with one of these phospholipids, rechromatography on silica adsorbent should have led to replacement of the monovalent metal ion by a divalent ion (31). The resulting divalent salt of cardiolipin would have been separated from the formerly comigrating phospholipid. As all three phospholipids gave a single spot upon rechromatographing (results not shown), we deduced that all cardiolipin molecules present in mitochondria lipids were localized in the band scraped after preparative TLC. That our cardiolipin preparations were free of any neutral lipid was guaranteed by the use of a first solvent mixture (see Materials and Methods), which moved all neutral lipids to the solvent front, several centimeters ahead of cardiolipin.

Fatty acid compositions of cardiolipins (Table 1) indicated that the level of saturated fatty acids was low in all of our preparations. This point deserves particular attention. As early as 1947, Pangborn (32) reported that cardiolipin isolated from beef heart did not contain saturated fatty acids. Since the advent of GLC, it appeared that saturated fatty acids were indeed present, but at very low levels (15). It then was suggested that the percentage of saturated acids perhaps was as good a criterion of purity as any other for cardiolipin (15). Results obtained by several authors with cardiolipin of different sources purified either by column, thin layer or high performance liquid chromatography (33–40) confirmed this. Based on these observations, it appears that any contamination of our cardiolipin preparations by another phospholipid should have resulted in higher

TABLE 1

Fatty Acid Composition[a] of Cardiolipin Extracted from Liver, Kidney and Heart Mitochondria of 21-day-old Male Wistar Rats

Fatty acid	Organ		
	Liver	Kidney	Heart
16:0	2.9 ± 0.4	3.7 ± 0.8	1.4 ± 0.3
16:1(n-7)	0.9 ± 0.4	1.1 ± 0.2	0.5 ± 0.1
18:0	0.7 ± 0.2	0.6 ± 0.3	0.4 ± 0.05
18:1(n-9)	2.4 ± 0.4	4.3 ± 0.3	2.6 ± 0.1
18:1(n-7)	8.2 ± 0.3	12.8 ± 0.8	10.8 ± 0.3
18:2(n-6)	78.4 ± 1.6	68.2 ± 3.4	61.8 ± 1.2
20:2(n-6)	2.3 ± 0.1	2.7 ± 0.1	2.0 ± 0.05
20:3(n-6)	0.9 ± 0.1	1.9 ± 0.1	4.5 ± 0.1
20:4(n-6)	1.2 ± 0.2	2.5 ± 0.2	4.9 ± 0.2
22:4(n-6)	t[b]	t	0.6 ± 0.1
22:5(n-6)	t	t	0.6 ± 0.1
22:5(n-3)	t	t	1.2 ± 0.2
22:6(n-3)	1.1 ± 0.2	0.6 ± 0.4	7.8 ± 0.8

[a]Composition as wt %. Values are means ± SE of analyses of four cardiolipin preparations for each organ.

[b]t = Trace amounts (less than 0.2%).

percentages of saturated fatty acids than reported in Table 1.

Table 1 indicates that the stearic acid level was similar in cardiolipins prepared from heart, liver and kidney mitochondria where it represented between 0.4 and 0.7% of total fatty acids. The level of palmitic acid was slightly higher and apparently organ-dependent: kidney cardiolipin contained ca. 3–4% of this fatty acid, liver cardiolipin between 2 and 3% and heart cardiolipin around 1.5%. Similar values have been reported for cardiolipins prepared from the same organs of adult rats (25), indicating that these characteristics could be age-independent, at least from weaning to adulthood. Other saturated fatty acids, including 14:0, 15:0, 17:0 and 20:0 acids, were also present, but each accounted for less than 0.1% of total fatty acids. Longer saturated fatty acids occasionally have been detected in noticeable amounts by others (41), but we were unable to confirm these findings.

Linoleic acid was the predominant unsaturated fatty acid in cardiolipin from organs of male Wistar rats taken at weaning: it represented from 62–78% of total fatty acids (Table 1), being less concentrated in heart than in kidney or liver cardiolipin. Similar percentages of linoleic acid have been determined in cardiolipin from whole heart of male Sprague-Dawley rats (42) and from whole liver of female Wistar rats (43) at weaning (56.4% and 78.5%, respectively). In adult male Wistar rats, cardiolipin from whole heart (44) or heart mitochondria (25) have a higher content of linoleic acid than cardiolipin from either liver (25,44) or kidney mitochondria (25).

The second important fatty acid in cardiolipin from the three organs was *cis*-vaccenic acid [18:1(n-7)]. This represented 75–80% of total octadecenoic acids, a proportion consistent with values reported for cardiolipins prepared from whole liver of adult rats (40) or beef heart (45). *cis*-Vaccenic acid also was the major octadecenoic acid in cardiolipin from adult rat heart or frog heart (Wolff, R. L., unpublished data). Inversely, the predominant octadecenoic acid in phosphatidylcholine and phosphatidylethanolamine from the same mitochondrial preparations was oleic acid [18:1(n-9)] (results not shown).

Small amounts of 20:2(n-6), 20:3(n-6), 20:4(n-6) and 22:6(n-3) acids were present in liver and kidney mitochondria cardiolipin. The sum of their percentages was 5.5% and 7.7%, respectively. In heart mitochondria cardiolipin, polyunsaturated fatty acids with more than 18 carbon atoms represented, altogether, 22% of total fatty acids. About one-third was docosahexaenoic acid (Table 1). High levels of arachidonic and docosahexaenoic acids also have been noticed in cardiolipin from heart of male Sprague-Dawley rats at weaning (42), but it seems that their levels in adult animals are lower (25,42). Although it has been suggested that these polyunsaturated fatty acids could originate from some contamination by other lipids (46), numerous authors have reported their presence in cardiolipin together with very low levels of saturated fatty acids (7,13,17, 24,33,35,39,40,42,44).

As our cardiolipins had reproducible low levels of saturated fatty acids, and more particularly very low percentages of stearic acid, we could deduce that contamination by other phospholipids was very low and that all fatty acids listed in Table 1 were integral parts of cardiolipin molecules. Consequently, our preparations were adequate substrates to study the positional distribution of fatty acids in cardiolipins.

Positional Distribution of Fatty Acids

De Haas et al. (5) have shown that snake venom phospholipase A_2 action on synthetic cardiolipin resulted in the release of 2 (2″) linked fatty acids exclusively. The commercial phospholipase A_2 used in the present study did not contain appreciable phospholipase A_1 activity, as could be judged from results obtained after incubation of phosphatidylethanolamine from rat liver mitochondria in the conditions of cardiolipin hydrolysis. The resulting lysophosphatidylethanolamine contained only 2.1% of polyunsaturated fatty acids, whereas the released fatty acids contained 2.8% of saturated acids.

Chemical hydrolysis of cardiolipin occurred in our conditions, but at a very low rate: in the absence of phospholipase A_2, less than 2% of cardiolipin were partially hydrolyzed after one hr of incubation (26). Because enzymatic hydrolysis also was complete after the same period (26), this side reaction was considered negligible. Moreover, we were unable to detect by TLC in hydrolysis products any compound corresponding to cardiolipin molecules which would have lost three fatty acids. This indicated that enzymatic hydrolysis of cardiolipin stopped when two fatty acids were released and that the resulting lysocardiolipin did not undergo further chemical degradation or significant acyl chain migration. From all these observations, it could be deduced that only fatty acids from the 2 (2″) positions of cardiolipin were released in our experimental conditions. That all of these fatty acids were released was

checked by TLC analysis of products formed at the end of the incubation period. No particular resistance to enzymatic hydrolysis of any cardiolipin preparation from the three studied organs was noticed contrary to previously reported observations (6).

The compositions of fatty acids esterified at positions 1 (1″) and 2 (2″) of cardiolipin from heart, liver and kidney mitochondria (Table 2) were obtained by GLC analysis of methyl esters prepared, respectively, from lysocardiolipin and free fatty acids after complete hydrolysis of cardiolipins.

Linoleic acid represented 89% of fatty acids esterified at the 1 (1″) positions and 67% at the 2 (2″) positions of cardiolipin from liver mitochondria (Table 2). In adult male Sprague-Dawley rats, corresponding values of 89.3% and 65.5% have been reported (10). A similar asymmetrical distribution also has been established in cardiolipin of whole liver from adult Charles River female rats (81.4% of linoleic acid at the 1 [1″] positions against 50.2% at the 2 [2″] positions) (7). It thus can be assumed that factors such as sex, age or strain of rats may not be responsible for the more random distribution of linoleic acid observed by other authors (6,8) in cardiolipin from rat liver.

Kidney cardiolipin also showed a marked asymmetrical distribution of linoleic acid (Table 2), which represented 87% of fatty acids at positions 1 (1″) and 51% at positions 2 (2″). Previous studies did not lead to such a clear-cut conclusion. In one case (6), only the leading edge of the cardiolipin peak eluted by column chromatography was studied, and thus, results did not apply to the entire cardiolipin population. In another report (9), linoleic acid was shown to be uniformly distributed between positions 1 (1″) and 2 (2″), but in this study, the percentage of saturated fatty acids in the cardiolipin preparation was high (17.2%) and indicative of some contamination or decomposition.

Imbalance of linoleic acid distribution was even more striking in cardiolipin from heart mitochondria than in cardiolipin from the two other organs: linoleic acid percentage at positions 1 (1″) was twice that at positions 2 (2″ (Table 2). No data were available for rat heart cardiolipin, but it has been shown that cardiolipin from human and beef heart (12) also exhibited an asymmetrical distribution of linoleic acid. This asymmetry was less marked than in the present study. Ratios of the percentage of linoleic acid esterified at the 1 (1″) positions and the 2 (2″) positions [1 (1″)/2 (2″) ratios] were 1.22 for human heart cardiolipin and 1.14–1.15 for beef heart (12) instead of 2.13 in this study. The reasons for this difference were that heart cardiolipin of rats at weaning contained considerably more polyunsaturated fatty acids with 20 or 22 carbon atoms than beef heart cardiolipin and that these fatty acids were mainly esterified at positions 2 (2″) (Table 2). In adult rat heart, these fatty acids are replaced by linoleic acid, which may reach 90% of total fatty acids (25,44,47). It thus may be assumed that

TABLE 2

Relative Composition of Fatty Acids[a] in the 1 (1″) and 2 (2″) Positions of Cardiolipin Isolated from Liver, Kidney and Heart Mitochondria of 21-day-old Male Wistar Rats

	Liver		Kidney		Heart	
Fatty acid	1 (1″)	2 (2″)	1 (1″)	2 (2″)	1 (1″)	2 (2″)
16:0	4.0 ± 1.2	1.5 ± 0.2	4.6 ± 0.3	1.8 ± 0.9	1.5 ± 0.1	1.4 ± 0.2
16:1(n-7)	0.5 ± 0.1	0.6 ± 0.05	1.1 ± 0.1	1.0 ± 0.2	0.7 ± 0.2	0.5 ± 0.1
18:0	1.4 ± 0.3	0.8 ± 0.1	0.7 ± 0.05	0.7 ± 0.05	0.4 ± 0.05	0.7 ± 0.1
18:1(n-9)	1.3 ± 0.1	3.8 ± 0.2	2.2 ± 0.1	6.2 ± 0.2	1.9 ± 0.2	3.7 ± 0.3
18:1(n-7)	0.6 ± 0.2	14.9 ± 0.3	1.1 ± 0.2	25.9 ± 0.8	1.4 ± 0.2	20.0 ± 0.9
18:2(n-6)	89.3 ± 2.1	67.8 ± 2.0	87.2 ± 1.0	51.2 ± 1.0	85.0 ± 0.8	39.8 ± 0.1
20:2(n-6)	0.4 ± 0.1	3.7 ± 0.1	0.2 ± 0.05	5.3 ± 0.3	0.5 ± 0.05	3.5 ± 0.1
20:3(n-6)	0.3 ± 0.1	1.3 ± 0.05	1.1 ± 0.1	2.8 ± 0.6	3.9 ± 0.2	5.6 ± 0.2
20:4(n-6)	0.3 ± 0.1	1.8 ± 0.05	0.4 ± 0.1	3.8 ± 0.8	1.9 ± 0.2	7.3 ± 0.1
22:4(n-6)	t[b]	t	t	t	0.2 ± 0.1	0.8 ± 0.2
22:5(n-6)	t	t	t	t	0.2 ± 0.1	1.0 ± 0.3
22:5(n-3)	t	0.3 ± 0.1	t	t	0.3 ± 0.1	1.7 ± 0.2
22:6(n-3)	0.2 ± 0.1	1.8 ± 0.1	0.1 ± 0.1	0.9 ± 0.3	2.0 ± 0.2	13.0 ± 0.7

[a]Composition as wt % of total fatty acids esterified at the indicated position. Values are means ± SE of duplicate analyses of products from two hydrolysis experiments in each case.

[b]t = Trace amounts (less than 0.2%).

heart cardiolipin of adult rats will resemble beef or human heart cardiolipin more closely with regard to the percentage of linoleic acid on each position.

Octadecenoic acids were nine- to 10-fold more concentrated at the 2 (2″) positions of cardiolipin from the three organs than at their 1 (1″) positions (Table 2). The percentages of *cis*-vaccenic acid were 3.1 to 5.4 times higher than those of oleic acid at positions 2 (2″) but slightly lower at positions 1 (1″). This is the first report of such a double asymmetrical distribution of octadecenoic acid isomers in cardiolipin molecules. Most previously reported data indicated that octadecenoic acids were esterified preferentially at positions 2 (2″) (6-10,12-14), but no details concerning the composition of isomers were given.

As in heart cardiolipin, polyunsaturated acids other than linoleic acid were esterified mainly at positions 2 (2″) of liver and kidney cardiolipin (Table 2).

Because stearic acid percentages were too low at both positions of cardiolipin (Table 2), it was not possible to draw any conclusion as to a preferential location of this fatty acid. But palmitic acid, at least in liver and kidney mitochondria, was localized predominantly in the 1 (1″) positions. Cardiolipin from whole liver of female Charles River rats (7) and from liver mitochondria of male Sprague-Dawley rats (10) also displayed the same asymmetrical distribution of palmitic acid.

From the present results, we can conclude that cardiolipins of mitochondria from rat organs show a highly selective positioning of unsaturated fatty acids. Positions 1 (1″) are esterified principally with linoleic acid and positions 2 (2″) with linoleic, octadecenoic and other polyunsaturated acids. But the question arises as to how this nonrandom arrangement evolves when the fatty acid composition of cardiolipin changes. Preliminary results obtained in our laboratory with cardiolipin prepared from liver of rats raised on a fat-free diet indicated that linoleic acid decrease was larger at positions 2 (2″) than at positions 1 (1″), but that all fatty acids remained asymmetrically distributed. The 1 (1″)/2 (2″) ratio for linoleic acid reached a value of 3.2, close to that reported for beef brain cardiolipin (2.7) (13). Thus, it seems that the higher affinity of linoleic acid for positions 1 (1″) of cardiolipin would be preserved when linoleic acid level in cardiolipin is naturally or experimentally low. These observations add further evidence against dependence of linoleic acid asymmetrical distribution in cardiolipin on the mammalian species or on the organ of origin of this phospholipid.

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METHODS

Thiobarbituric Acid Reaction of Aldehydes and Oxidized Lipids in Glacial Acetic Acid

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ABSTRACT

Thiobarbituric acid (TBA) reaction of several aldehydes and oxidized lipids in glacial acetic acid was performed. All the samples were freely soluble in the solvent used. Saturated aldehydes produced a stable yellow pigment with an absorption maximum at 455 nm, a red pigment derived from malonaldehyde at 532 nm, and an orange pigment due to dienals at 495 nm. The absorbance maximum was 7–9 per μmol for saturated aldehydes, 27.5 per μmol for malonaldehyde and about 2 per μmol for dienals. Autoxidation of unoxidized lipids increased progressively in glacial acetic acid. When the TBA test was performed under nitrogen, autoxidation of unoxidized lipids was inhibited completely. While saturated aldehydes produced no yellow pigment under nitrogen, oxidized lipids produced a considerable amount of stable yellow pigment. The value for absorbance at 455 nm as a function of autoxidation time paralleled those of peroxide values. The absorbance of most oxidized lipids at 455 nm was higher than at 532 nm. Yellow pigment formation in the TBA test under nitrogen could not be ascribed to free saturated aldehydes but rather to unspecified closely related substances. The stable yellow pigment was found to be an excellent indicator of lipid oxidation.

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INTRODUCTION

The TBA test generally is used for determination of lipid oxidation. TBA produces a red pigment with an absorption maximum at 532 nm by reaction with malonaldehyde formed during lipid oxidation (1,2). However, the oxidation of polyunsaturated fatty acids has been shown to give various aldehydes besides malonaldehyde (3,4). Several reports have discussed the reaction of TBA with various saturated and unsaturated monofunctional aldehydes. Jacobson et al. (5) reported that the reaction between TBA and a saturated aldehyde in a single-phase solvent of *iso*-octane, 1-propanol and water produced a yellow pigment with an absorption maximum at 452 nm, while the reaction between TBA and a dienal produced a red pigment with a maximum at 532 nm. Marcuse and Johansson (6) demonstrated that the reaction of a saturated aldehyde in water produced a yellow pigment with a maximum at 450 nm. Measurement of the yellow pigment for determination of lipid rancidity is limited, because the pigment is very unstable in an aqueous medium. Thus, the yellow pigment generally is recognized as an interfering component for measurement of red pigment (7–9).

We investigated the reaction of several aldehydes and oxidized lipids with TBA in glacial acetic acid and found that saturated aldehydes and oxidized lipids produced extensive amounts of the stable yellow pigment. In this paper, we describe the use of this yellow pigment for measurement of lipid oxidation.

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MATERIALS AND METHODS

Materials

TBA, glacial acetic acid (special grade), butylated hydroxytoluene (BHT), 1-propanal, 1-butanal and 1-hexanal were the products of Wako Pure Chemical Industries Ltd. (Osaka, Japan). Malonaldehyde bis(dimethylacetal), 1-heptanal, 1-propanal dimethylacetal, 2-ethyl-2-hexenal, *trans*-2-hexenal, methyl oleate and methyl linoleate were obtained from Tokyo Kasei Kogyo Company Ltd. (Tokyo, Japan). 2,4-Hexadienal and *trans,trans*-2,4-decadienal were from Aldrich Chemical Co. Inc. (Milwaukee, Wisconsin). Purified malonaldehyde sodium salt was prepared as described elsewhere (10). Linoleic acid 13-hydroperoxide (13-LOOH) was prepared enzymatically (11) from pure linoleic acid (Sigma Chemical Co. Ltd., St. Louis, Missouri).

Purified lipids of hog, beef and chicken were prepared by warming and pressing the corresponding subcutaneous fats obtained commercially and washing with hot distilled water several times. Lipids of sardine, beef liver and beef muscle were extracted as follows. The

sample was mixed with an equal volume of 1.15% KCl and homogenized; the homogenate then was mixed with an equal volume of methanol and two vol of chloroform and centrifuged at 3000 rpm for 10 min. The chloroform/methanol layer was collected and evaporated to dryness.

Autoxidation of Lipids

Methyl oleate, methyl linoleate and purified lipids from hog, beef and chicken tissues were oxidized at 98 C by the active oxygen method (AOM) (12) for 70 hr. Chloroform/methanol extracts of the tissue were dissolved in benzene (2.0 g/20 ml) and placed in culture dishes (diameter 10 cm). Samples then were irradiated at a distance of 30 cm from two Toshiba U.V. lamps at 325 nm (Toshiba Company Ltd., Tokyo, Japan) at room temperature for 70 hr. Solvent was supplied intermittently to replace that lost by evaporation. The peroxide value of each oxidized lipid was determined according to the Wheeler method (13).

TBA Reaction

An indicated amount of each lipid sample, which included the standard aldehyde, was dissolved in 5.0 ml of 0.4% TBA/glacial acetic acid in a test tube with a screw cap. The mixture was heated at 100 C either in air or under nitrogen gas for up to five hr. After cooling, the absorption spectrum of the clear reaction mixture was measured with a UV-200S Shimadzu double beam spectrophotometer or a UV-240 UV-visible Shimadzu recording spectrophotometer.

RESULTS

Reaction of Various Aldehydes with TBA in Glacial Acetic Acid

The saturated and unsaturated aldehydes as well as malonaldehyde and their acetals were reacted with TBA in glacial acetic acid at 100 C. 1-Hexanal produced a yellow pigment showing a single absorption maximum at 455 nm. Characteristics of the formation of the yellow pigment are shown in Figure 1. The absorbance at 455 nm increased gradually up to five hr. The relationship between the amount of 1-hexanal and the absorbance at 455 nm was linear after the five hr reaction period (Fig. 1 insert). The presence of 2% BHT suppressed formation of the yellow pigment by about 40%. The reaction in the presence of 20% water yielded only 10% yellow pigment. This lower yield could be due to the lability of the yellow pigment in the aqueous medium. The pigment produced in glacial acetic acid after three hr of reaction was shown to be labile in aqueous media by demonstrating that it was degraded progressively by subsequent addition of water. When the reaction was performed under nitrogen, yellow pigment formation was inhibited completely. A reaction system that had been bubbled with nitrogen gas and subsequently aerated produced yellow pigment to the same extent as that in the same system under air, indicating the aldehyde was not purged by bubbling. The aldehyde pretreated in glacial acetic acid at 100 C for five hr also produced no yellow pigment in subsequent reaction with TBA under nitrogen, indicating that the yellow pigment formation was not due to oxidation of the aldehyde in glacial acetic acid.

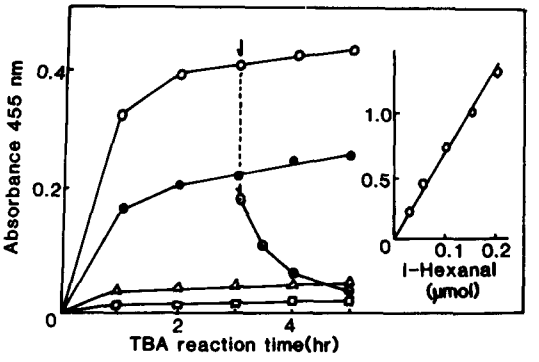

FIG. 1. Time-course of yellow pigment formation in reaction of 1-hexanal with TBA. 1-Hexanal (0.05 μmol) was dissolved in 5.0 ml of TBA/glacial acetic acid (○), TBA/glacial acetic acid containing 2% BHT (●) and TBA/water-glacial acetic acid (1:4, v/v) (△), and the mixtures were heated at 100 C. As indicated by an arrow, 1.0 ml of water was added (⊙). A mixture of 1-hexanal in TBA/glacial acetic acid was gassed with nitrogen and similarly heated (□). The insert indicates a calibration curve of 1-hexanal treated with TBA/glacial acetic acid at 100 C for 5 hr.

Other saturated aldehydes, 1-propanal, 1-butanal, and 1-heptanal, showed the same type of reaction with TBA. The amounts of yellow pigment as estimated by absorbance at 455 nm were between 7–9 per μmol aldehyde (Table 1). When two aldehydes were mixed and reacted with TBA, the absorbance value observed was the sum of that obtained for each aldehyde. 1-Propanal dimethylacetal produced yellow pigment under air but not under nitrogen. The yield of yellow pigment under air was similar to that for 1-propanal (Table 1). The reaction of the free and acetal forms of the aldehyde were quite similar.

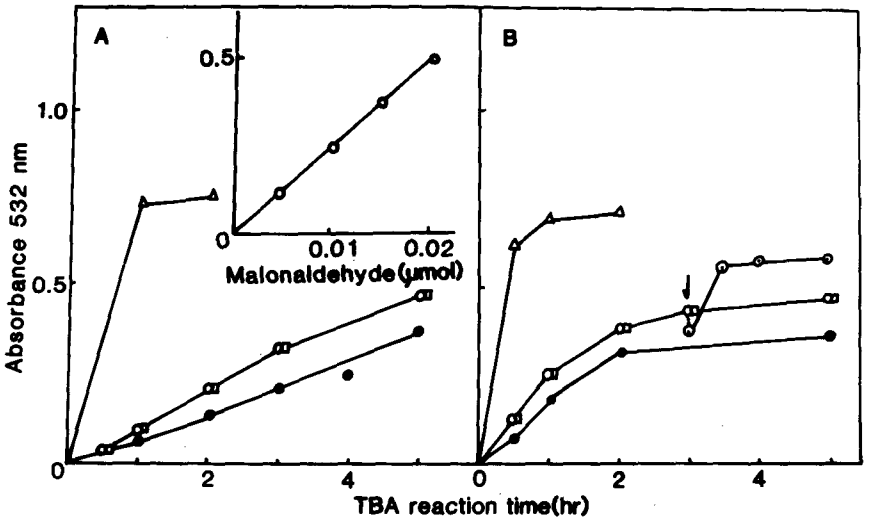

FIG. 2. Time-course of red pigment formation in reaction of free malonaldehyde (A) and malonaldehyde bis(dimethylacetal) (B) with TBA. Malonaldehyde sodium salt or malonaldehyde bis(dimethylacetal) (0.02 μmol) was dissolved in 5.0 ml of TBA/glacial acetic acid (○), TBA/glacial acetic acid containing 2% BHT (●) and TBA/water-glacial acetic (1:4, v/v) (△), and the mixtures were heated at 100 C. As indicated by an arrow, 1.0 ml of water was added (⊙). A mixture of malonaldehyde sodium salt or malonaldehyde bis(dimethylacetal) in TBA/glacial acetic acid was gassed with nitrogen and similarly heated (□). The insert indicates a calibration curve of malonaldehyde treated with TBA/glacial acetic acid at 100 C for 5 hr.

TABLE 1

Absorbance of the Reaction Mixture of the Aldehyde in 5 ml of 0.4% TBA/Glacial Acetic Acid at 100 C for 5 Hr

Aldehyde	Absorbance/μmol aldehyde		
	455 nm	495 nm	532 nm
1-Propanal	7.4		
1-Butanal	8.2		
1-Hexanal	8.2		
1-Heptanal	9.0		
1-Propanal dimethylacetal	7.4		
Malonaldehyde sodium salt			27.5
Malonaldehyde bis(dimethylacetal)			27.5
trans-2-Hexenal	0.4		
2-Ethyl-2-hexenal	0.1		
2,4-Hexadienal	1.2	2.5	1.0
trans,trans-2,4-Decadienal	2.1	2.0	1.0

Malonaldehyde sodium salt and malonaldehyde bis(dimethylacetal) produced a red pigment with a single absorption maximum at 532 nm, both having a similar absorbance intensity (Fig. 2). The relationship between the amount and absorbance at 532 nm was linear (Fig. 2A insert). The absorbance at 532 nm per μmol free malonaldehyde or malonaldehyde bis(dimethylacetal) was 27.5 (Table 1); this value was about four times as large as that at 455 nm obtained from the saturated aldehydes. Addition of water to the reaction mixtures increased the formation of red pigment about two-fold. The presence of BHT suppressed the formation of red pigment from both the free and acetal forms. In contrast to the reaction of the saturated aldehydes, this reaction was not influenced when carried out under nitrogen.

Monoenals, *trans*-2-hexenal and 2-ethyl-2-hexenal, produced a yellow pigment with a single absorption maximum at 455 nm, but the absorbance per μmol of aldehyde was extremely low (Table 1). Dienals, 2,4-hexadienal and *trans,trans*-2,4-decadienal, produced an orange pigment with three absorption maxima at 455, 495 and 532 nm, with the absorbance at 495 nm being the highest (Fig. 3A). The absorbance at 455 and 532 nm was much lower than that of the saturated aldehydes and malonaldehyde, respectively (Table 1). Formation of the orange pigment was greatly suppressed under nitrogen and by water (Fig. 3A). The lower yield of the orange pigment in aqueous acetic acid could not be ascribed to the lability of the pigment, since

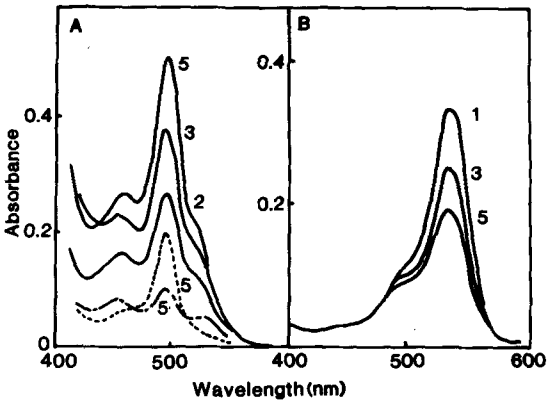

FIG. 3. Absorption spectra of the reaction mixtures of 2,4-hexadienal with TBA. A: 2,4-Hexadienal (0.2 μmol) was heated at 100 C in TBA/glacial acetic acid (———), TBA/glacial acetic acid-water (4:1, v/v) (— - —) and TBA/glacial acetic acid gassed with nitrogen (- - - -). B: 2,4-Hexadienal (1 μmol) was heated at 100 C in TBA/water. Numerals indicate time (hr) of reaction.

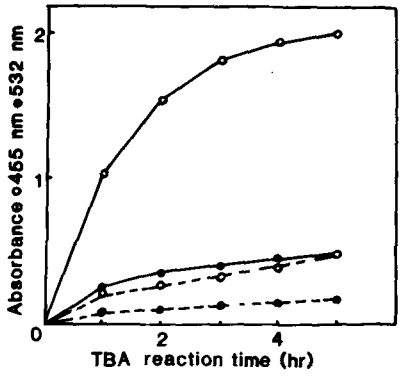

FIG. 4. Time-courses of yellow and red pigment formation during reaction of 13-LOOH with TBA. 13-LOOH (1.0 mg, 3.2 μmol) was dissolved in TBA/glacial acetic acid and the mixture was heated at 100 C (———). The mixture was gassed with nitrogen and heated at 100 C (- - - -).

the pigment produced in glacial acetic acid was not destroyed by subsequent addition of water. It is interesting to note that when the dienals were treated with TBA in water alone, a red pigment having a single absorption maximum at 532 nm was produced (Fig. 3B). Although the absorption spectrum of the red pigment was indistinguishable from that of the pigment derived from malonaldehyde, they were different in stability. Thus, the red pigment from 2,4-hexadienal was destroyed progressively in water (Fig. 3B), while the red pigment from malonaldehyde was stable.

Reaction of Linoleic Acid 13-Hydroperoxide with TBA in Glacial Acetic Acid

Reaction of 13-LOOH with TBA in glacial acetic acid at 100 C showed three absorption maxima at 455, 495 and 532 nm, with the absorbance at 455 nm being the highest. 13-LOOH may be degraded into a complex mixture of compounds (3,4) under the reaction conditions. The time-course of increase in absorbance at 455 and 532 nm is shown in Figure 4. The increase in absorbance at 532 nm probably is due primarily to malonaldehyde, which was estimated to be 0.015 μmol (0.5% of the hydroperoxide) after reaction for five hr. The reaction was suppressed under nitrogen to 40% of the control value, and may reflect retarded degradation of the hydroperoxide. The increase in absorbance at 455 nm might be due to the saturated aldehydes produced by degradation of the hydroperoxide. The amount of saturated aldehydes liberated was estimated to be 0.24 μmol (7.5%) after reaction for five hr. Yellow pigment formation was suppressed to about 25% under nitrogen. Formation of the yellow pigment under nitrogen was high in spite of the retarded degradation of the hydroperoxide and the unreactivity of the saturated aldehydes (Fig. 1). Yellow pigment formation from the hydroperoxide under nitrogen could not be due to the free saturated aldehydes but, rather, to other closely related substances.

Reaction of Oxidized Methyl Oleate and Linoleate with TBA in Glacial Acetic Acid

When unoxidized methyl oleate and linoleate were treated with TBA in glacial acetic acid at 100 C, absorbance at 455 and 532 nm increased progressively, indicating that the esters were oxidized during TBA reaction. Addition of 2% BHT prevented the autoxidation, but not completely. Treatment of unoxidized methyl esters with TBA under nitrogen produced no yellow or red pigments. Thus, oxidation during the TBA reaction was inhibited completely under nitrogen. Therefore, measurement of the extent of oxidation of lipids must be performed under nitrogen.

Methyl oleate and linoleate were oxidized by the AOM, and the oxidized esters were reacted with TBA in glacial acetic acid at 100 C for five hr under nitrogen. As oxidation of the esters proceeded, the reaction mixtures with TBA revealed three absorption maxima at 455, 495 and 532 nm. Relationships between the amount

of the oxidized esters and absorbance at 455 nm (Fig. 5 insert) and at 532 nm were linear. Figure 5 shows the time-course of absorbance at 455 and 532 nm against the AOM time of the esters. Absorbance at 455 nm was much higher than at 532 nm throughout the oxidation time of the esters. Profiles of absorbance at 455 nm were correlated roughly to the peroxide values of both the oxidized esters. The absorbance at 455 nm may be an excellent indicator of peroxidation of these esters. Formation of the pigment could not, however, be ascribed to free saturated aldehydes, but instead to closely related substances. The increase in absorbance at 532 nm was lower than that at 455 nm with both oxidized esters. Absorbance at 532 nm was extremely low with the oxidized methyl oleate throughout the AOM time.

Reaction of Oxidized Fats and Oils with TBA in Glacial Acetic Acid

Hog, beef and chicken fats, which had been oxidized by the AOM, were reacted with TBA in glacial acetic acid at 100 C for five hr under nitrogen. The time-course of the peroxide value and the absorbances at 455 and 532 nm against AOM time are shown in Figure 6. It was found

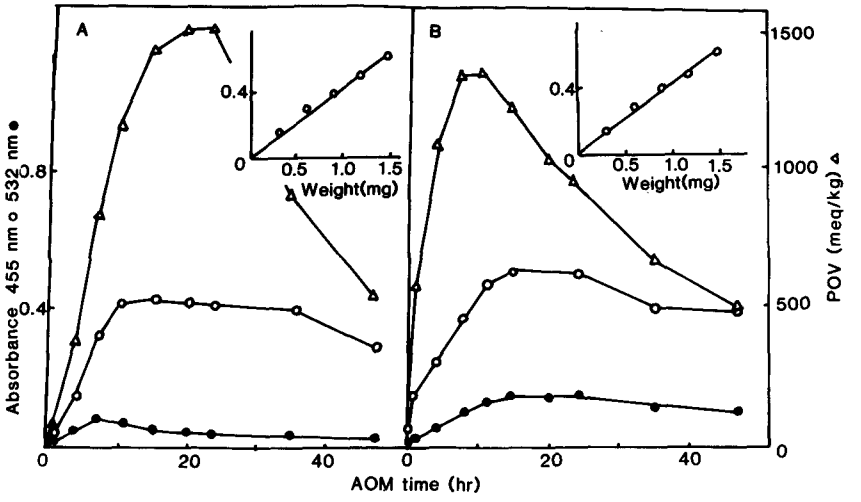

FIG. 5. Time-courses of yellow and red pigment formation in oxidized methyl oleate (A) and methyl linoleate (B). Methyl oleate or methyl linoleate was autoxidized by the AOM, and the oxidized ester (1.0 mg) was reacted in TBA/glacial acetic acid at 100 C for 5 hr under nitrogen. Absorbance at 455 and 532 nm was plotted against AOM time. Peroxide values also were plotted. Inserts indicate calibration curves of absorbance at 455 nm of the oxidized esters (AOM time: 20 hr).

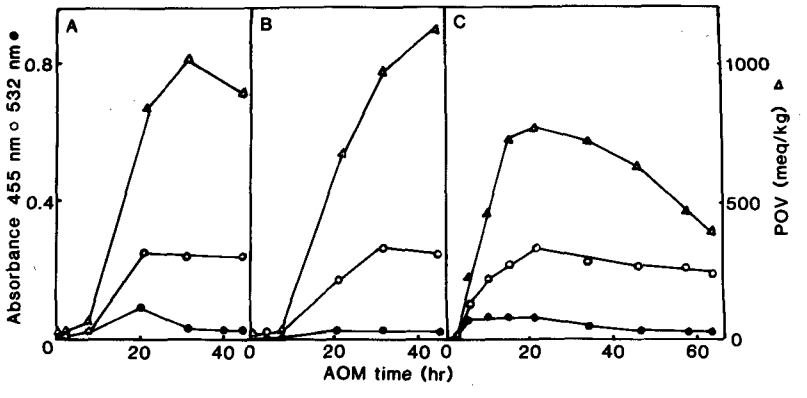

FIG. 6. Time-courses of yellow and red pigment formation in oxidized hog (A), beef (B) and chicken (C) fat. Hog, beef or chicken fat was autoxidized by the AOM, and the oxidized fat (1.0 mg) was reacted with TBA as described in Fig. 5.

that absorbance at 455 nm was more intense than at 532 nm under these conditions, and paralleled the increase and decrease in peroxide values.

Chloroform/methanol extracts of sardine, beef liver and beef muscle, which had been oxidized by ultraviolet irradiation, were reacted with TBA. The time-course of the peroxide value, and the absorbances at 455 and 532 nm as a function of ultraviolet irradiation time of the extract, are shown in Figure 7. The absorbance at 455 nm showed a parallel increase and decrease with the peroxide values. The absorbance at 455 nm was more intense than the absorbance at 532 nm except for sardine extract. The sardine extract showed a higher absorbance at 532 nm, which may be due to its higher polyunsaturated fatty acid content.

DISCUSSION

The TBA test generally is used for measurement of oxidation of lipids. It forms a red pigment with an absorption maximum at 532 nm by reaction with malonaldehyde and its precursors (1,2). Aqueous acidic solutions have been used in the TBA tests reported so far. It was found in the present experiments that deterioration of lipids could be monitored sensitively at 455 nm when the TBA test is carried out in glacial acetic acid.

Performing the TBA reaction in glacial acetic acid had advantages. Glacial acetic acid could solubilize all the lipid samples for the test. Most procedures described in the literature were performed in aqueous acidic media, and lipids are not completely solubilized, possibly interfering with spectrophotometric measurement of the red pigment. For instance, unsolubilized lipid has been removed by extraction (14) or by centrifugation (15) before spectrophotometric measurement. It is doubtful whether a reaction in which not all lipid is solubilized in the reaction mixture accurately reflects the degree of oxidation of the samples. It was found in the present experiment that the formation of red pigment from malonaldehyde and malonaldehyde bis(dimethylacetal) in glacial acetic acid was only about half that obtained in the aqueous acetic acid (Fig. 2).

The yellow pigment produced by the reaction of the saturated aldehydes with TBA was stable in glacial acetic acid under the reaction conditions. Jacobson et al. (3) and Marcuse and Johansson (6) demonstrated that a saturated aldehyde produced a yellow pigment in the reaction with TBA in aqueous media, but the pigment was very unstable. Therefore, the yellow pigment has been considered an interfering product for measurement of the red pigment (7–9). Under the present conditions, the saturated aldehydes produced a stable yellow pigment, which could be measured by its absorbance at 455 nm. The absorbance at 455 nm obtained with saturated aldehydes was about one-fourth of that in 532 nm from malonaldehyde or malonaldehyde bis(dimethylacetal) (Table 1).

The dienals produced an orange pigment with an absorption maximum at 495 nm on reacting with TBA in glacial acetic acid. The dienals produced a different type of pigment in glacial acetic acid, although they produced the red pigment in water (5,6). But the absorbance at 495 nm was much lower than that at 455 nm from the saturated aldehydes, and also lower than that at 532 nm observed for malonaldehyde and malonaldehyde bis(dimethylacetal).

Oxidation of lipids during the TBA reaction in glacial acetic acid increased progressively. 13-LOOH was degraded during the reaction in glacial acetic acid (Fig. 4). Some investigators have used antioxidants such as BHT to prevent autoxidation of lipids during TBA reaction in aqueous acidic media (15,16). It was found in the present experiments that BHT could not inhibit completely autoxidation of unoxidized methyl oleate and linoleate during TBA reaction in glacial acetic acid. Furthermore, BHT influenced formation of the yellow and red pigments from the saturated aldehydes and malonaldehyde (Figs. 1 and 2). Autoxidation of unoxidized lipids during the TBA reaction in glacial acetic acid was inhibited completely under nitrogen. Therefore, it is recommended that measurement of the degree of lipid peroxidation with TBA in glacial acetic acid be performed under nitrogen. It is interesting to note that while formation of the red pigment from malonaldehyde and its acetal was affected little under nitrogen (Fig. 2), formation of the yellow pigment from saturated aldehydes and their acetals was inhibited dramatically under nitrogen (Fig. 1). The reason for this effect is not known. The yellow pigment formed in the reaction of oxidized lipids with TBA (Figs. 5, 6 and 7) cannot be derived from the saturated aldehydes but from other closely related products. Nevertheless, the increase in absorbance at 455 nm always was higher than the increase at 532 nm throughout the oxidation process of lipids except for sardine lipid. Thus, absorbance at 455 nm was a better indicator of oxidized lipids than that at 532 nm.

It has been demonstrated that the yellow pigment also was produced during the reaction of TBA with sugars and other water-soluble impurities (17–20). The specificity of the reaction

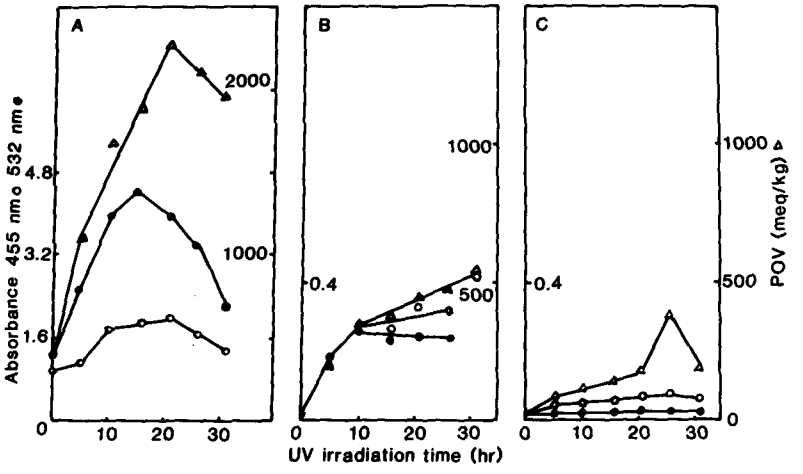

FIG. 7. Time-courses of yellow and red pigment formation in oxidized chloroform/methanol extract of sardine (A), beef liver (B) and beef muscle (C). The chloroform/methanol extract was oxidized by ultraviolet irradiation, and the oxidized extract (1.0 mg) was reacted with TBA as described in Fig. 5.

for lipids can be maintained, however, if the lipid is isolated by extraction with organic solvents prior to the assay. The lipids used in the present experiment were free of these contaminants, and the absorbance at 455 nm can be ascribed to some unspecified species derived from lipid oxidation.

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Yuko Chiba provided technical assistance.

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Separation of Isomeric Lysophospholipids by Reverse Phase HPLC

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ABSTRACT

A reverse-phase high performance liquid chromatography (HPLC) method was developed which resolved isomers of lysophosphatidylcholine (LPC) differing in the location of the aliphatic chain (*sn*-1 or *sn*-2 position) and the position (Δ^6 or Δ^9) or geometric configuration (*cis* or *trans*) of the olefin group in monounsaturated species. LPC isomers containing an acyl substituent at the *sn*-2 position eluted before their 1-acyl-*sn*-glycero-3-phosphocholine (1-acyl LPC) counterparts. The retention times of both the *sn*-1 and *sn*-2 isomers of monounsaturated species increased in the order Δ^9-*cis* < Δ^9-*trans* < Δ^6-*cis*. The integrated ultraviolet absorbance (203 nm) in binary mixtures of the Δ^9-*cis* and Δ^6-*cis* 2-acyl lysophospholipid isomers correlated with the lipid phosphorus content of corresponding column eluates ($r = 0.994$). Thus, the present method will facilitate synthesis of isomerically pure diradylphospholipids by providing homogeneous lysophospholipid precursors and help simplify the quantitative analysis of unsaturated lysophospholipid species.
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INTRODUCTION

Isomerically pure diradylphospholipids labeled exclusively at the *sn*-1 or *sn*-2 position are required as chemical probes to investigate the metabolism and physical properties of homogeneous molecular species of phospholipids. Preparation of a specifically labeled homogeneous diradylphospholipid by acylation of a lysophospholipid precursor requires the use of an isomerically pure starting material. Isolation of homogeneous lysophospholipid species derived from biologic sources has been achieved recently by reverse-phase HPLC for saturated mono-acyl species (1) and for alkyl-ether and vinyl-ether derivatives (2). However, no method has been described that resolves positional (Δ^6 or Δ^9) or geometrical (*cis* or *trans*) isomers of unsaturated lysophospholipid species. The present method was developed to provide a convenient means for isolating isomeric lysophospholipids based upon the location and stereochemistry of the unsaturated center. This reverse-phase HPLC method allows quantitative recovery of lysophospholipids without significant migration of the acyl group during chromatography, in contrast to the substantial migration that occurs with conventional methods employing silicic acid as the stationary phase. Furthermore, measurement of the UV absorption at 203 nm during the course of separation allowed direct quantitation of monounsaturated lysophospholipid isomers. Thus, this method should prove useful in the synthesis of homogeneous molecular species of phospholipids and in providing a basis for a simplified approach to the structural characterization of diradylphospholipids from biologic sources.

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MATERIALS AND METHODS

HPLC

All separations were performed using a Waters HPLC system consisting of a Model 720 system controller, Model 730 printer-plotter-integrator, Model 6000A pump and Model U6K injector. Elution profiles were obtained by measuring UV absorbance at 203 nm with a Model 450 UV detector equipped with an 8 μl flow cell after injection of samples dissolved in 10-250 μl of MeOH/$CHCl_3$ (1/1, v/v). All separations were performed using a stainless steel column (4.6 mm × 25 cm) packed with 5 μ C-18 derivatized spherical porous silica particles (Ultrasphere ODS, Altex, Berkeley, California) and a precolumn (3.2 mm × 7 cm) packed with a C-18 bonded phase on 30 μm glass beads (Co:Pell ODS, Whatman, Clifton, New Jersey) at room temperature (20-24 C). Individual LPC isomeric species were eluted isocratically using a mobile phase comprised of methanol/water/acetonitrile (57:23:20, v/v/v) containing 20 mM choline chloride. Flow rate was maintained at 2 ml/min which resulted in an operating pressure of 2000-4000 psi.

Solvents

Chloroform and water were obtained from Burdick and Jackson (Muskegon, Michigan);

acetonitrile and methanol were Omnisolve solvents obtained from VWR (St. Louis, Missouri). Choline chloride was purchased from Sigma (St. Louis, Missouri). Solvents were filtered through a 0.45 μm silicon filter (Millipore) and degassed thoroughly under vacuum prior to use.

Lipids

Synthetic dioleoyl-PC (1,2-*cis*-9-octadecenoyl-*sn*-glycero-3-phosphocholine), dipetroselinoyl-PC (1,2-*cis*-6-octadecenoyl-*sn*-glycero-3-phosphocholine) and dielaidyl-PC (1,2-*trans*-9-octadecenoyl-*sn*-glycero-3-phosphocholine) were obtained from Avanti (Birmingham, Alabama). All standards produced a single spot following thin layer chromatography (TLC) on Silica Gel G after exposure of the developed plate to I_2 vapor. Fast atom bombardment mass spectra of each standard demonstrated a prominent peak at m/z = 786 ($[MH]^+$) without evidence of other PC molecular species.

1-Monoacyllysophospholipid species were prepared by enzyme-catalyzed hydrolysis of homogenous diacylphospholipids using phospholipase A_1 (PLA_2) (*Naja Naja* venom, Sigma) as described previously (3). 2-Monoacyllysophospholipids were prepared by the method of Slotboom et al. (4) using lipase from *Rhizopus arrhizus* (phospholipase A_1 [PLA_1], Sigma). Reaction mixtures were extracted twice at 0–4 C under N_2 by the method of Bligh and Dyer (5). The combined chloroform layers then were dried under N_2 and resuspended in $CHCl_3$/MeOH (1:1, v/v). Lysophospholipids were separated conveniently from diacylphospholipids and free fatty acids present in the reaction mixture by injecting the extracted lipids onto a 4.6 mm × 7.5 cm stainless steel column packed with 10 micron Ultrapack-ODS (Beckmann-Altex, Berkeley, California). LPC was eluted within five min, using the mobile phase described previously at a flow rate of 1 ml/min. Diacylphospholipids were eluted with 100% methanol. The entire procedure required 20 min.

Phosphate Analysis, Fatty Acid Esterification, Gas Chromatography and Mass Spectrometry

Lipid phosphorus in column eluates was determined by the method of Chen (6) after preliminary ashing with 10% $MgNO_3$ in MeOH as described by Ames (7). Preparation of fatty acid methyl esters (FAME) as well as methods for capillary gas chromatography (GC) and fast atom bombardment mass spectroscopy (FABMS) have been described previously (3).

Calculation of Relative Retention Time (RRT)

RRT values for individual molecular species were adjusted to account for the time required to displace the void volume using the formula:

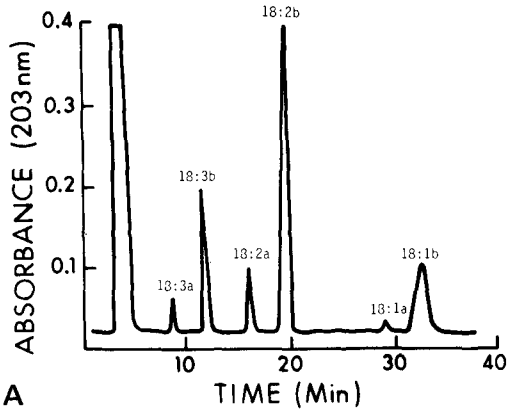
$$RRT = \frac{(\text{retention time of component species} - t_o)}{(\text{retention time of internal standard} - t_o)}$$

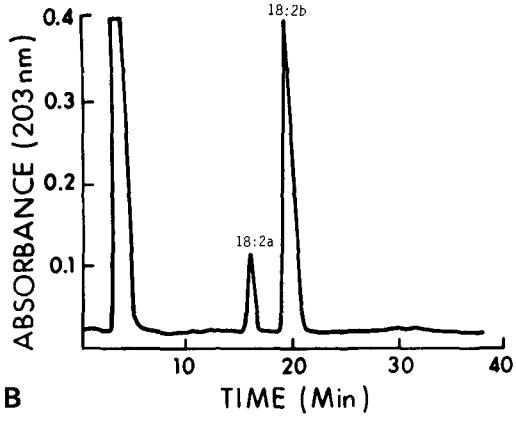
where t_o = time required to elute the solvent front peak.

RESULTS

In initial experiments, soybean LPC molecular species were separated by reverse-phase HPLC as described (Fig. 1A). The FAME derivatives prepared from column eluates corresponding to peaks 18:3a and 18:3b were identified as esters of 18:3 fatty acid by the parent ions from electron impact mass spectroscopy (m/z = 292). Similarly, peaks 18:2a and 18:2b produced methyl esters of 18:2 fatty acid after derivatization as ascertained by the parent ion obtained from electron impact mass spectroscopy (m/z = 294). Peaks 18:3b and 18:2b were identified tentatively as the 1-acyl isomers of LPC containing 18:3 and 18:2 fatty acids, respectively, while peaks 18:3a and 18:2a were assigned tentatively to the 2-acyl-*sn*-glycero-3-phosphocholine (2-acyl LPC) isomers containing 18:3 and 18:2 esterified fatty acids based on the following evidence: (a) The 18:3 FAME derivatives from peaks 18:3a and 18:3b were indistinguishable by comparison of their GC retention times on a 30 m capillary GC column and their complete fragmentation patterns obtained from electron-impact mass spectroscopy. Similarly, the 18:2 FAME derivatives from peaks 18:2a and 18:2b had identical GC elution profiles and electron impact fragmentation patterns. (b) HPLC separation of aliquots of the column eluate corresponding to peak 18:2b after incubation in the presence or absence of silicic acid (shown in Figs. 1B and 1C) demonstrated that silicic acid catalyzed the interconversion of these isomeric LPC species under conditions that do not lead to migration of double bonds or *cis-trans* isomerization. (c) The ratio of the integrated UV absorbance for peaks 18:3b/18:3a and peaks 18:2b/18:2a from Figure 1A were each approximately equal to a value of 8 which is close to the previously reported value of 9 for the equilibrium ratio of 1-acyl/2-acyl isomers of palmitoyl-LPC (8).

To characterize further the ability of reverse-phase HPLC to resolve isomeric lysophospholipid species, we prepared 1-acyl and 2-acyl LPC isomers by regiospecific enzyme-catalyzed





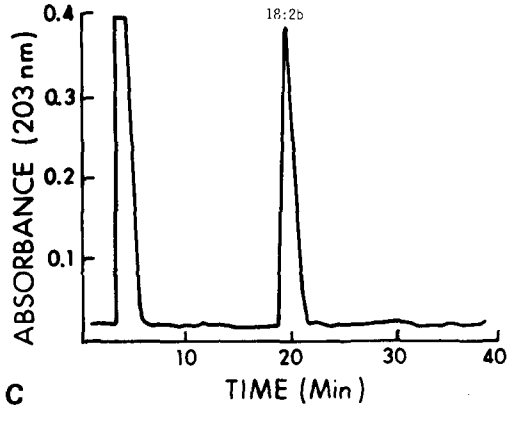


FIG. 1. A. Separation of molecular species of soybean LPC. 1.4 μmol of LPC were dissolved in 100 μl $CHCl_3$/MeOH (1:1, v/v) and injected onto a 25 cm × 0.46 cm column of 5 μm Ultrasphere-ODS. Individual components were eluted isocratically at room temperature (20–24 C) with a flow rate of 2 ml/min, using a solvent mixture comprised of methanol/water/acetonitrile (57:23:20, v/v/v) containing 20 mM choline chloride. UV absorbance was measured at 203 nm (0.2 AUFS); operating pressure was 3400 psi. Individual peaks were identified as described in Materials and Methods. Peaks are designated according to the composition of the acyl chain (i.e., length: number of double bonds). The letters *a* and *b* refer to the 2-acyl LPC and 1-acyl LPC isomers, respectively. B. The column eluate corresponding to peak 18:2b in Fig. 1A was extracted with $CHCl_3$ as described in Materials and Methods, dried under N_2, resuspended in 100 μl $CHCl_3$/MeOH (2:1, v/v) containing 5 mg of silicic acid and incubated for 12 hr at room temperature. The solution was filtered, dried and separated using the chromatographic conditions described for Fig. 1A. Peaks are designated as described for Fig. 1A. C. The column eluate corresponding to peak 18:2b in Fig. 1A was extracted, dried and resuspended as described for Fig. 1B. The purified lysophospholipid was incubated for 12 hr at room temperature in the absence of silicic acid. Peaks are designated as described for Fig. 1A.

hydrolysis of symmetric phosphatidylcholine molecular species containing 18:1 fatty acids that differed only in the position (Δ^6 versus Δ^9) or geometry (*cis* versus *trans*) of the unsaturated group in the acyl chain. Following separation of the reaction mixtures, column eluates containing the 1-acyl and 2-acyl LPC isomers were collected and analyzed by FABMS. All six LPC isomers produced identical parent ions $(MH)^+$ (m/z = 522). Representative chromatograms of the LPC species in reaction mixtures following hydrolysis of 1,2-*cis*-9-octadecenoyl-*sn*-glycero-3-phosphocholine with PLA_1 and PLA_2 are shown in Figures 2A and 2B, respectively. Although the PLA_2-catalyzed reaction produced an isomerically pure lysophospholipid product (see Fig. 2B), the reaction catalyzed by PLA_1 resulted in small amounts of contamination of the 2-acyl LPC product with the thermodynamically favored 1-acyl LPC isomer (see Fig. 2A). Similarly, PLA_2-catalyzed hydrolysis of 1,2-*trans*-9-octadecenoyl-*sn*-glycero-3-phosphocholine or 1,2-*cis*-6-octadecenoyl-*sn*-glycero-3-phosphocholine produced isomerically pure products. However, when these compounds were hydrolyzed by PLA_1, the 2-acyl LPC products also were contaminated with small amounts of the 1-acyl LPC isomers. Because previous work has

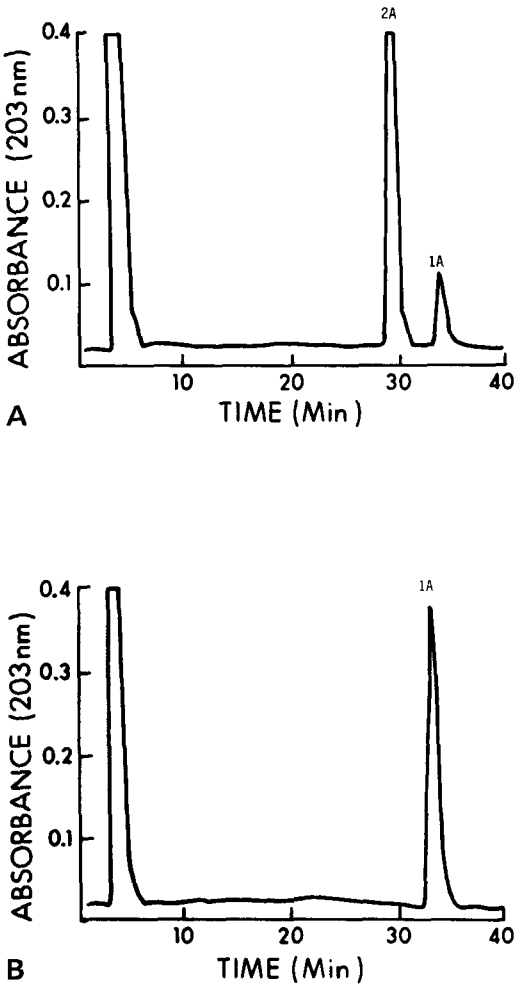


FIG. 2. A. Chromatogram of LPC species produced by PLA_1-catalyzed hydrolysis of dioleoyl-PC. Dioleoyl-PC was incubated with PLA_1 from *Rhizopus arrhizus* in 0.5 M borate buffer (pH = 6.5) containing 5 mM $CaCl_2$ for 2 hr at room temperature. Reaction products were extracted with $CHCl_3$/MeOH as described in Materials and Methods and separated using the conditions given for Fig. 1A. The peak designations correspond to those given in Table 1. B. Chromatogram of LPC species produced by PLA_2-catalyzed hydrolysis of dioleoyl-PC. Dioleoyl-PC was incubated with PLA_2 from *Naja Naja* snake venom in 20 mM Tris buffer (pH = 7.4) containing 10 mM $CaCl_2$ for 20 min at room temperature. Reaction products were extracted as described in Materials and Methods and separated using the chromatographic conditions given for Fig. 1A. The peak designation corresponds to that given in Table 1.

demonstrated that the equilibrium mixture of palmitoyl LPC isomers is comprised of 90% 1-acyl and 10% 2-acyl species (8), then the rate constant for acyl migration from the *sn*-2 to the *sn*-1 position is nine times greater than that for migration in the reverse direction. Accordingly, the longer incubation time necessary for the PLA_1-catalyzed hydrolysis in combination with the higher rate constant for spontaneous acyl migration likely accounts for the observed distribution of LPC product species. However, we could not definitely exclude the possibility of minute amounts of PLA_2 contamination.

To determine the elution sequence and RRTs of individual species of 1- and 2-acyl LPC isomers, aliquots of column eluates corresponding to 1-acyl LPC isomers were collected from all three PLA_2 reaction mixtures and combined; 1-linoleoyl-LPC (peak 2B of Fig. 1) was added as an internal standard, and the mixture was separated as described. The retention time increased when the double bond was closer to the polar headgroup and the stereochemistry of the olefin group was of the *trans* configuration (Fig. 3). The elution sequence observed for the *cis* and *trans* isomers corresponded to that described previously for the reverse-phase HPLC separation of FAME and acetates of long chain fatty alcohols (9). However, it should be noted that considerable improvement in resolution of geometric isomers was achieved by the present method.

The same procedure was applied to column eluates obtained from PLA_1-catalyzed hydrolysis of isomeric PC. The results

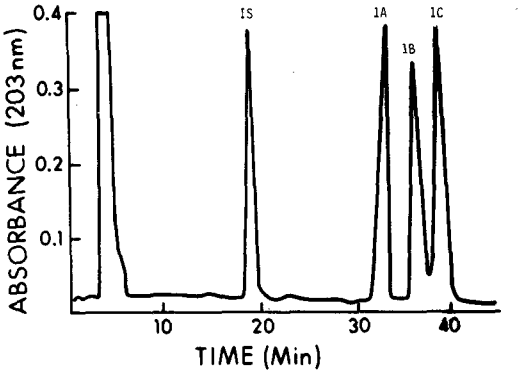


FIG. 3. Separation of 1-acyl LPC isomers prepared by PLA_2-catalyzed hydrolysis of symmetric PC molecular species. Aliquots of the reaction mixtures obtained following hydrolysis of dioleoyl-PC, dipetroselinoyl-PC and dielaidyl-PC (prepared as described for Fig. 2B) were combined and the LPC product species separated as described for Fig. 1A. IS = Internal standard (1-linoleoyl LPC). Other peak designations correspond to those given in Table 1.

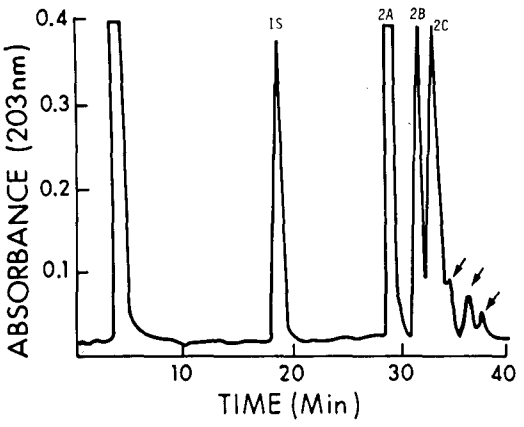


FIG. 4. Separation of 2-acyl LPC isomers prepared by PLA_1-catalyzed hydrolysis of symmetric PC species. Aliquots of the reaction mixtures obtained following hydrolysis of dioleoyl-PC, dipetroselinoyl-PC and dielaidyl-PC (prepared as described for Fig. 2A) were combined and the components separated as described for Fig. 1A. Peak designations correspond to those given in Table 1. Arrows identify peaks representing the 1-acyl LPC isomers as described in Results.

demonstrated an identical elution sequence for the different 2-acyl LPC isomers (Fig. 4). The small peaks indicated by arrows likely represent trace amounts of the 1-acyl LPC species present as a result of acyl migration that occurred at pH = 6.5 during the two hr reaction period. When individual 2-acyl LPC isomers were isolated from the reaction mixtures by HPLC, they could be stored in $CHCl_3$ at 0–4 C for up to 36 hr with acyl migration resulting in the formation of <1% of the 1-acyl species. Acyl migration did not occur during chromatographic resolution of the LPC regioisomers because reinjection of column eluates corresponding to either the 1-acyl or 2-acyl LPC species produced a chromatogram containing a single peak eluting at the expected retention time.

Table 1 shows the elution sequence and gives RRT values for each LPC species. Table 2 presents the ratio of RRT values for pairs of isomers that differ by a single modification in molecular structure. From the values of the ratios given, it can be seen that movement of the acyl chain from the *sn*-2 to the *sn*-1 position had the greatest effect on retention time, leading to a 20–25% increase in the RRT value. Separation of the 6-*cis* and 9-*trans* 18:1 LPC isomers was very sensitive to alterations in mobile phase composition, particularly with respect to changes in the aqueous component. It thus was necessary to make small adjustments in the composition of the mobile phase to achieve optimal resolution whenever a new column was used.

TABLE 1

Relative Retention Times (RRTs) of Isomeric 18:1 Lysophosphatidylcholine (LPC) Species

Peak designation[a]	LPC molecular species		RRT[b]
	Fatty acid	Position	
2A	9-*cis*-octadecanoic	*sn*-2	1.42
2B	9-*trans*-octadecanoic	*sn*-2	1.60
2C	6-*cis*-octadecanoic	*sn*-2	1.68
1A	9-*cis*-octadecanoic	*sn*-1	1.76
1B	9-*trans*-octadecanoic	*sn*-1	1.93
1C	6-*cis*-octadecanoic	*sn*-1	2.00

[a]Listed in order of elution from the column. Peak designations correspond to those shown in Figs. 2–4.

[b]RRT values were determined as described in Materials and Methods using 1-linoleoyl LPC (corresponding to peak 18:2b in Fig. 1A) as internal standard.

In addition to providing isomerically pure compounds for synthetic purposes, the present method was examined for potential quantitative analysis of positional and geometrical isomers of unsaturated fatty acids esterified to diradylphospholipids. Binary mixtures of 2-acyl lysophospholipid isomers containing *cis* 18:1 fatty acid (6-*cis* or 9-*cis*) in varying relative amounts were prepared. The concentration of 9-*cis* 18:1 LPC in all mixtures was much greater than that of 6-*cis* 18:1 LPC, to simulate the relative amounts of these isomers that would be encountered in phospholipid mixtures from biologic sources. The integrated UV detector response (λ = 203 nm) for each peak was compared to the amount of lipid phosphorus in the corresponding column eluate. Results demonstrated a strong correlation (r = 0.994) between the values obtained by the independent analyses (Fig. 5).

DISCUSSION

The recent introduction of C-18 derivatized porous silica microparticles (<10 μm) for reverse-phase HPLC has facilitated the resolution of individual molecular species of diradylphospholipids and lysophospholipids with only subtle differences in the structural constitution of the aliphatic side-chain(s). For example, Patton and coworkers (10) were able to separate individual diacylphospholipid species according to chain length and degree of unsaturation of the esterified aliphatic groups. More recently, Nicholas et al. (1) were able to

TABLE 2

Effects of Altering Molecular Structure on Relative Retention Time (RRT)

Isomer type	Distinguishing structural feature	Peak designation[a]		Isomer 1/Isomer 2 RRT ratio[b]
		Isomer 1	Isomer 2	
		sn-1	*sn*-2	
Regioisomers	Acyl-chain location	1A	2A	1.24
		1B	2B	1.21
		1C	2C	1.20
		6-*cis*	9-*cis*	
Positional isomers	Double bond position	2C	2A	1.18
		1C	1A	1.14
		9-*trans*	9-*cis*	
Geometrical isomers	Double bond geometry	2B	2A	1.13
		1B	1A	1.10

[a]Peak designations correspond to those given in Table 1.

[b]RRT values were determined as described in Materials and Methods using 1-linoleoyl LPC (corresponding to peak 18:2b in Fig. 1A) as internal standard.

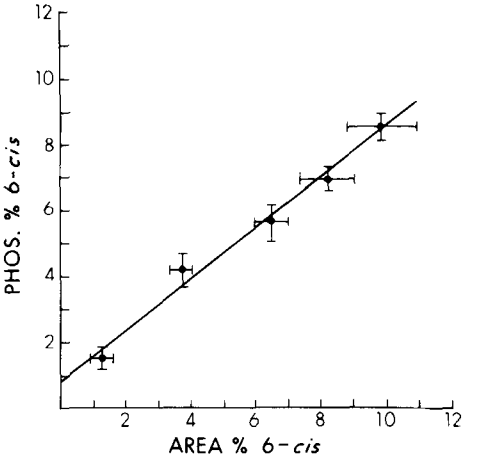


FIG. 5. Comparison of methods for the quantitation of monounsaturated LPC species following HPLC separation. Binary mixtures comprised of 90–99% 1-oleoyl LPC and 1–10% 1-petroselinoyl LPC (by mass) were prepared and the components separated by reverse-phase HPLC as described for Fig. 1A. Area % refers to results obtained by integrating the UV detector response for each isomer (i.e., area % = 100 × 1-petroselinoyl LPC peak area/total area). Phos. % refers to the results of phosphate measurements from the corresponding column eluates (i.e., phos. % = 100 × nmol 1-petroselinoyl LPC/total nmol injected). Values shown represent the mean ± SD of three determinations from five different binary mixtures. From linear regression analysis (n = 15): r = 0.9940, slope = 0.865, and y-intercept = 0.807.

separate lysophospholipids according to the location of the aliphatic side-chain (i.e., *sn*-1 or *sn*-2 position) using reverse-phase HPLC. They reported that the separation of 1-acyl and 2-acyl isomers was possible only when μ-Bondapak C-18 was used as the stationary phase; however, we have been able to obtain separation of isomeric lysophospholipids utilizing Ultrasphere-ODS, Econosphere C-18 and Lichrospher C-18 packings. With our HPLC system, the Ultrasphere-ODS column reproducibly provided the greatest separation efficiency (40,000–50,000 plates/meter compared to 25,000–35,000 plates/meter for the other two stationary phases). The marked improvement in resolution of the 1-acyl and 2-acyl LPC isomers utilizing the present method most likely is due to the addition of acetonitrile and choline chloride to the mobile phase, as well as the use of 5 μm porous silica particles as the stationary phase. The 20–25% difference in retention times for the 1-acyl and 2-acyl lysophospholipid species achieved by the present method allowed the isolation of preparative amounts (20–30 mg) of LPC utilizing a 1 cm × 25 cm column. In addition to providing complete separation of the 1-acyl and 2-acyl LPC isomers, the method described is capable of resolving unsaturated lysophospholipids according to the position and configuration of the double bonds in the aliphatic side-chain. For monounsaturated LPC species, the sensitivity of UV detection is determined by the extinction coefficient of the olefin group and samples containing only 5–10 nmol of a single molecular

species of monounsaturated LPC are quantified easily.

Previously available methods for identifying positional and geometrical isomers of esterified fatty acids in phospholipids utilized capillary GC alone or in tandem with mass spectrometry to characterize the volatile products produced after phospholipase treatment, transesterification, and oxidation of the double bond with ozone, permanganate-periodate or other oxidants (11). The present method potentially could facilitate the characterization of geometrical and positional isomers of diradylphospholipids by permitting a simplified alternative that is rapid and quantitative. When combined with regiospecific enzyme-catalyzed hydrolysis using PLA_1 and PLA_2, this method will provide a useful adjunct to conventional techniques for the structural characterization of individual species in complex mixtures of diradylphospholipids derived from biologic sources.

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COMMUNICATIONS

Effect of Very Long Chain Fatty Acids on Peroxisomal β-Oxidation in Primary Rat Hepatocyte Cultures

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ABSTRACT

Previous studies have demonstrated that certain high fat diets can induce peroxisomal fatty acid β-oxidation in rodent liver and that this may be due to their content of *trans* 22:1 fatty acids. In this study we have examined the effects of *cis* and *trans* 22:1 fatty acids (erucic and brassidic) and oleic acid (18:1) on palmitoyl-CoA oxidation, carnitine acetyltransferase and carnitine palmitoyltransferase activities in primary rat hepatocyte cultures. Brassidic and erucic acid and, to a lesser extent, oleic acid were cytotoxic to rat hepatocytes. However, at a concentration of 0.1 mM, brassidic acid produced small increases in palmitoyl-CoA oxidation and carnitine acetyltransferase activities in hepatocytes cultured 70 hr. Treatment of cells with 0.1 and 0.3 mM of either erucic or oleic acid had no effect on any of the enzymes measured.
Lipids 20:929–932, 1985.

INTRODUCTION

A number of chemicals, including the hypolipidemic drug clofibrate [ethyl 2-(p-chlorophenoxy) isobutyrate] and the plasticizer di-(2-ethylhexyl)-phthalate, have been shown to produce hepatic peroxisome proliferation and induction of peroxisomal fatty acid β-oxidation in rodents (1–3). The increase in peroxisome numbers and associated enzyme activities also have been demonstrated in vitro with primary rat hepatocyte cultures (4,5).

Apart from xenobiotics, nutritional factors such as fasting (6), high fat diets (7–9), vitamin E deficiency (10) and diabetes (11) have been shown to increase peroxisomal β-oxidation in the rat. In studies with different dietary oils, partially hydrogenated fish and rapeseed oils were found to increase peroxisomal β-oxidation to a greater extent than soybean oil (12). Furthermore, it has been suggested (12) that the content of *trans* 22:1 fatty acids in partially hydrogenated fish and rapeseed oils is responsible for the stimulation of peroxisomal fatty acid oxidation in rats fed these oils. In order to examine this hypothesis, we have studied the effects of *cis* and *trans* 22:1 fatty acids (erucic and brassidic) and, for purpose of comparison, oleic acid on peroxisomal β-oxidation and certain other enzyme activities in primary rat hepatocyte cultures.

METHODS

Hepatocytes were isolated from male Sprague-Dawley rats (Olac [1976] Ltd., Blackthorn, Bicester, Oxon, United Kingdom) (body wt 180–220 g) by a collagenase perfusion technique described previously (5). Viability, determined by trypan blue exclusion, was in the range 80–95%. Hepatocytes were seeded at 2.5×10^6 viable cells per three ml culture medium (RPMI 1640 containing 5% fetal calf serum, 50 μg/ml gentamicin, 1 μM insulin and 0.1 mM hydrocortisone-21-sodium succinate) in 60 mm petri dishes and maintained at 37 C in a humidified atmosphere of 5% CO_2/95% air. Exposure to fatty acids was commenced after two hr by replacing the culture medium with medium containing the fatty acids. Subsequently, the medium was changed and the cells were redosed every 24 hr.

Fatty acids initially were dissolved in serum-free culture medium containing 5% fatty acid-free bovine serum albumin (BSA) by stirring at 50 C and adding the minimum quantity of NaOH required to achieve a solution. These stock solutions (approximately 2–5 mM) were sterilized by passage through a 0.22 μm filter. After determination of the actual fatty acid concentrations by gas liquid chromatography (GLC) (12), stock solutions were diluted with culture medium adjusted to give the correct final concentrations of the additions stated above. Control cultures were treated with medium containing the same final concentrations of BSA.

For biochemical determinations, the cell monolayers were washed with 0.154 M KCl/50 mM Tris-HCl pH 7.4, dispersed in this buffer (1.25 ml per dish), and homogenized by sonication. Cyanide-insensitive palmitoyl-CoA oxidation (5) and protein (13) were measured in fresh cell

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homogenates. Carnitine acetyltransferase and carnitine palmitoyltransferase activities were measured in once freeze-thawed cell homogenates as described previously (4,5). Cytotoxicity was assessed by morphological examination of the cultures and measurement of lactate dehydrogenase activity (assayed at 37 C) released into the culture medium (14). Statistical analysis of the data was performed using Dunnett's test for multiple comparisons (15).

RESULTS AND DISCUSSION

Brassidic and erucic acids were overtly toxic to cultured rat hepatocytes when added to the culture medium at concentrations of 0.5 and 0.8 mM, respectively. Both acids produced extensive cell death and disruption of the cell monolayer within 24 hr, although the effects of brassidic acid were more severe than erucic acid. Cultures maintained for up to 96 hr with 0.1 or 0.2 mM brassidic acid showed a time- and concentration-dependent increase in cell death; surviving cells appeared enlarged, with a more granular cytoplasm than the controls (Figs. 1A, 1B). Similar but slightly less marked changes were produced by 0.32 mM erucic acid; oleic acid was comparatively nontoxic, with concentrations up to 0.44 mM having little effect on cell morphology. Consistent with the morphological observations, 24 hr treatment with both 0.20 mM brassidic and 0.32 mM erucic acid produced a marked increase in lactate dehydrogenase release into the culture medium, whereas 0.44 mM oleic acid produced less effect (Table 1).

Because of the observed cytotoxicity of the fatty acids, concentrations of ≤0.3 mM oleic and erucic acids and 0.1 mM brassidic acid were used to investigate effects on peroxisomal β-oxidation, carnitine acetyltransferase and palmitoyltransferase activities. As previously observed, a marked decrease in palmitoyl-CoA oxidation was observed in hepatocytes cultured 70 hr compared to 0 hr cultured cells (5,16), whereas the activities of carnitine acetyltransferase and carnitine palmitoyltransferase were less affected (Table 2). At the concentrations examined, none of the fatty acids had any effect on the latter enzyme activity, localized in mitochondria (7). Brassidic, but not oleic and erucic acids, stimulated (compared to 70 hr control cells) the activities of palmitoyl-CoA oxidation and carnitine acetyltransferase (Table 2). The latter enzyme is found in peroxisomal, mitochondrial and microsomal fractions (7,17). As a positive control, rat hepatocytes also

TABLE 1

Leakage of Lactate Dehydrogenase from Rat Hepatocyte Cultures Treated with Fatty Acids

Hepatocyte preparation[a]	Enzyme activity (nmol/min/ml medium)
Control cells cultured 24 hr	713 ± 13[b]
Cells cultured 24 hr with:	
Oleic acid (0.44 mM)	863 ± 41[c]
Erucic acid (0.32 mM)	1000 ± 12[d]
Brassidic acid (0.20 mM)	1302 ± 58[d]

[a]All cells were cultured in the presence of 1.2% BSA.

[b]Values are expressed as mean ± SEM of 3-4 dishes.

[c]Significantly different (Dunnett's test) from control cells, $p < 0.05$.

[d]Significantly different (Dunnett's test) from control cells, $p < 0.01$.

TABLE 2

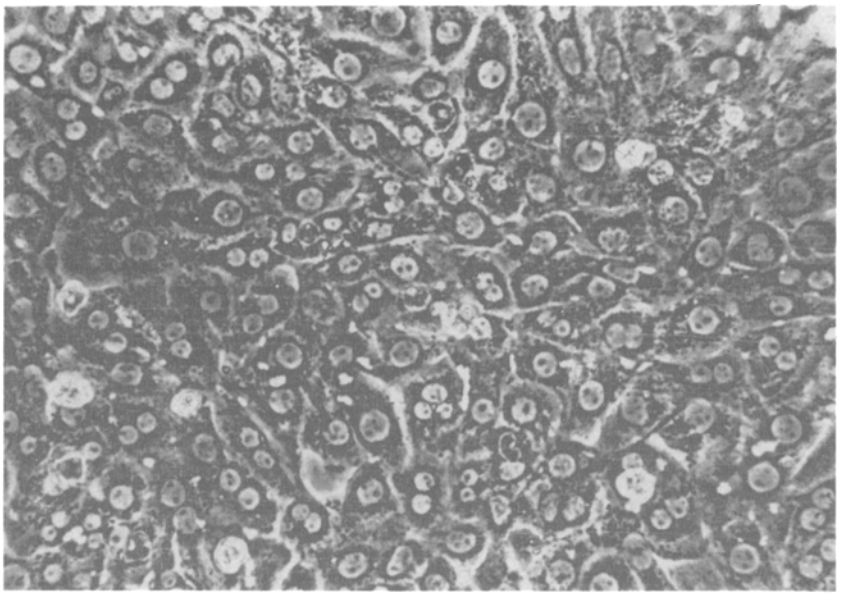
Effects of Fatty Acids and Clofibric Acid on Enzyme Activities in Rat Hepatocyte Cultures

	Enzyme activity (nmol/min/mg protein)[b]		
Hepatocyte preparation[a]	Palmitoyl-CoA oxidation	Carnitine acetyltransferase	Carnitine palmitoyltransferase
0 hr cultured cells	3.16 ± 0.13	3.03 ± 0.40	7.11 ± 0.18
Control cells cultured 70 hr	0.61 ± 0.07	1.90 ± 0.29	9.12 ± 1.00
Cells cultured 70 hr with:			
Oleic acid (0.1 mM)	0.70 ± 0.04	2.88 ± 0.32	8.84 ± 0.80
Oleic acid (0.3 mM)	0.78 ± 0.05	2.81 ± 0.18	8.82 ± 0.78
Erucic acid (0.1 mM)	0.65 ± 0.06	2.27 ± 0.20	7.16 ± 0.72
Erucic acid (0.3 mM)	0.80 ± 0.11	2.90 ± 0.22	7.93 ± 1.20
Brassidic acid (0.1 mM)	1.10 ± 0.06[c]	3.42 ± 0.40[c]	8.53 ± 0.92
Clofibric acid (0.5 mM)	8.92 ± 0.37[c]	34.1 ± 1.9[c]	18.3 ± 1.6[c]

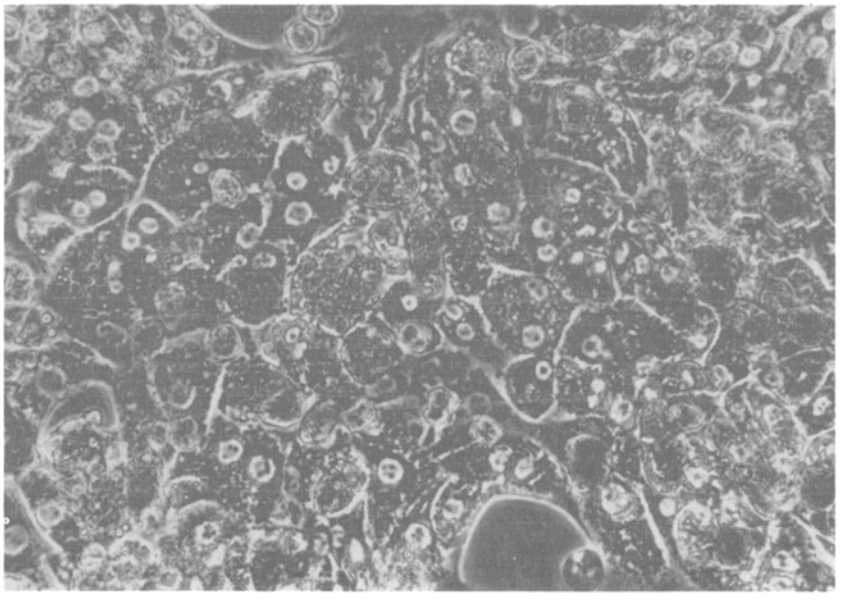
[a]All 70 hr treated cells were cultured in the presence of 0.36% BSA.

[b]Values are expressed as mean ± SEM of 4-6 dishes. Results of a typical experiment are shown.

[c]Significantly different (Dunnett's test) from 70 hr control cells $p < 0.01$.



A



B

FIG. 1. A = Control culture 70 hr; normal appearance of hepatocyte monolayer with only occasional dead cells. B = Culture treated with 0.2 mM brassidic acid for 70 hr; extensive cell death with disruption of the monolayer. Live cells enlarged with granular cytoplasm. Enlargements 450×.

were cultured with clofibric acid, the primary metabolite of clofibrate in several species (18). Clofibric acid addition produced the expected large increases in palmitoyl-CoA oxidation and carnitine acetyltransferase activities (19) and also doubled carnitine palmitoyltransferase activity (Table 2).

In these studies, the cytotoxicity of the fatty acids prohibited relatively high concentrations from being tested in vitro for induction of peroxisomal enzyme activities. However, brassidic acid did produce a small stimulation of palmitoyl-CoA oxidation and carnitine acetyltransferase, both of which have been shown to be increased in the livers of rats fed high fat diets (7,12,20). The results of these in vitro studies with brassidic and oleic acids are consistent with in vivo findings that feeding rats partially hydrogenated rapeseed or fish oils (rich in very long chain monoenoic fatty acids) resulted in an induction of peroxisomal enzyme activities, whereas soybean oil (rich in C18 fatty acids) had little effect (12,20). With respect to the increase in palmitoyl-CoA oxidation, the present results could represent a stabilization of enzyme activity, rather than de novo protein synthesis, due to the fall in enzyme activity which occurs in cultured rat hepatocytes (5,16). Further studies are required to establish whether the observed stimulation in cell culture represents a true induction of peroxisomal proteins.

ACKNOWLEDGMENTS

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Highly Saturated Lipid Composition of Ctenophore Cilia: Possible Indication of Low Membrane Permeability

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ABSTRACT

The lipids of ciliary membranes in two species of ctenophore are found to be distinguished by phospholipids containing short chain saturated fatty acids and by saturated sterols. Such a composition is expected to result in a much lower permeability of cilia membranes to water and other ions than the general body membranes. We suggest that this is related to the large surface area of the cilia and is a requirement, on the part of the ctenophore, to control water and ionic fluxes across its surface membranes.
Lipids 20:933-935, 1985.

INTRODUCTION

The properties of cell membranes are affected profoundly by their lipid composition and, hence, it is to be expected that the fatty acid and sterol composition of particular membranes will be adapted to their specific roles. Previous work on tunicates (1-3) has indicated that ciliary membranes have a lipid composition quite distinct from membranes found elsewhere in the animal, i.e., particularly rich in saturated sterols and fatty acids. This unusual lipid composition may contribute to specific structural or permeability requirements of the ciliary membrane (2,3).

It is normally difficult to obtain uncontaminated samples of cilia, free from the cells bearing them. Previously we have successfully isolated fragments of cilia from the ascidian tunicate *Ciona*. The larger ctenophores, however, offer exceptional material for the study of ciliary membranes, since they use large comb-plates composed of many very long cilia for propulsion.

MATERIALS AND METHODS

Fresh specimens of *Leucothoe harmata* and *Beroe ovata* were taken by hand-scoop net in the Bay of Villefranche, South France. Cilia from individual comb-plates were snipped from the basal cells with fine scissors. The ciliary fragments, basal cells plus remaining cilia (it was not possible to obtain a sample of the basal cells free from cilia), and the body were extracted separately with chloroform/methanol (4). The component lipids then were analyzed quantitatively for the major lipid classes by Chromarod-flame ionization detection techniques, and the phospholipids and sterols separated on a preparative scale (1). Detailed fatty acid and sterol analyses were performed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) techniques (1). Each sample was analyzed at least three times; the results expressed represent an average of these analyses. The results have an estimated error range of the order of ±2% for the most abundant components and ±0.3% for minor components.

RESULTS AND DISCUSSION

Lipids of the cilia and basal cells were dominated by phospholipids (>80% total lipid), with sterols as a significant component (5-10% total lipid). The body lipids also were dominated by phospholipids (>60%) with smaller amounts of sterols, diglycerides and triglycerides and traces of hydrocarbons. The phospholipids of the *Beroe* cilia and the basal cells plus cilia show a distinctly different fatty acid composition from the body phospholipids (Table 1). Short chain (C_{14}-C_{18}) saturated acids predominate in the samples containing ciliary membranes, while long chain (C_{18}-C_{22}) polyunsaturated acids were major components of the body phospholipids. Of particular note were the high levels (5-8% total fatty acid) of 15:0 acid in the former; this acid is not normally a major component of biological membranes. A similar pattern of fatty acid distribution was seen for the *Leucothoe* samples (Table 1). The sterol data for the *Beroe* samples (Table 2) show the cilia to contain a considerably higher relative percentage of more saturated sterols ($27\Delta^5$, $29\Delta^5$) compared to the body ($26\Delta^{5,22}$, $27\Delta^{5,22}$, $27\Delta^5$, $28\Delta^{5,22}$).

The results from the ctenophore ciliary membranes studied here are similar to those

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TABLE 1

Fatty Acid Composition of the Samples (Expressed as % of Total Fatty Acids)

	Beroe			Leucothoe		
Fatty acid	Cilia phospholipids	Basal cell phospholipids	Body phospholipids	Cilia phospholipids	Basal cell phospholipids	Body phospholipids
12:0	ND[a]	ND	ND	7.9	9.3	5.3
14:0	18.9	17.3	14.6	17.5	15.2	11.0
Iso 15:0	—	—	0.3	0.9	1.4	0.7
Anteiso 15:0	—	—	0.3	0.9	1.4	0.7
15:0	8.0	5.1	1.3	9.5	7.8	3.7
16:2	—	—	—	—	—	2.2
16:1	1.4	2.6	3.0	2.2	T[b]	2.3
16:0	38.7	47.4	17.1	34.9	35.0	29.6
Iso 17:0	—	—	T	T	T	0.7
Anteiso 17:0	—	—	T	T	T	1.4
17:0	—	—	2.2	2.5	2.5	2.6
18:4	—	—	1.7	—	—	—
18:2	—	—	3.6	0.6	1.9	2.2
18:1	19.8	12.8	4.3	5.9	5.5	4.6
18:0	13.2	13.5	9.4	14.0	13.6	13.1
20:5	—	1.3	8.4	0.8	1.8	4.8
20:1	—	—	1.3	0.6	0.8	0.7
20:0	—	T	T	0.3	1.4	2.1
22:6	—	T	28.2	0.4	1.5	7.9
22:1	—	—	4.3	0.4	0.4	T
22:0	—	—	T	0.4	0.6	4.1

[a]ND = not determined.
[b]T = <0.3%.

TABLE 2

Sterol Composition of the Samples (Expressed as % of Total Sterol)

	Beroe		
Sterol	Cilia	Basal cells	Body
$26\Delta^{5,22}$	3.7	11.7	10.2
$26\Delta^{22}$	1.5	0.7	T[a]
$26\Delta^{5}$	T	1.1	0.4
$27\Delta^{5,22}$ occel.	3.7	12.8	12.0
$27\Delta^{5,22}$	4.5	12.4	16.2
$27\Delta^{22}$	T	—	—
$27\Delta^{5}$	23.9	16.6	18.7
$27\Delta^{0}$	T	1.5	2.0
$27\Delta^{5,24}$	T	1.1	1.6
$28\Delta^{5,22}$	4.8	15.8	17.6
$28\Delta^{22}$	T	T	0.8
$28\Delta^{5,24[28]}$	1.7	3.4	6.6
$28\Delta^{5}$	1.6	1.5	1.2
$29\Delta^{5,22}$	1.1	1.1	1.8
$29\Delta^{5}$	44.8	17.7	6.0
$29\Delta^{0}$	T	—	—
$29\Delta^{5,24[28]}$	6.3	3.4	3.2
$29\Delta^{24[28]}$	0.5	T	0.1
$30\Delta^{5,24[28]}$	0.7	0.6	0.5

[a]T = <0.1%.

previously obtained from the tunicate *Ciona* (3). The lipids of the ciliary membranes in all three species are distinguished by phospholipids with short chain saturated fatty acids and by saturated sterols.

The precise role, if any, of lipids in determining the permeability of biological membranes remains uncertain. It has been shown repeatedly for many poikilotherms that there is an inverse relationship between environmental temperature and the unsaturated fatty acid content of the organism's membrane lipids (5–15). It is believed that these changes are related to adjustment of plasma membranes fluidity. In the case of fatty acids, the saturated and unsaturated carbon chains have different configurations. Saturated acids have a relatively straight chain configuration, while unsaturated acids have a kinked chain which is particularly pronounced in acids with normal *cis*-double bonds. This kinking of the carbon chain makes close association between neighboring molecules difficult and, hence, interferes with the establishment of strong van der Waal interactions (8). Consequently, membranes with a high content of unsaturated fatty acids with normal *cis*-double bonds tend to

have a more disrupted lipid array and be more fluid (16). The degree of fluidity is important in determining the functional activity of a membrane, e.g., permeability (7). Almost certainly other membrane components such as sterols, which can have varying degrees of ethylenic unsaturation in both the ring and side chain, also will influence membrane lability and permeability (17,18).

Our results indicate that the ciliary membranes in tunicates and ctenophores, by having saturated fatty acids in the phospholipids and corresponding types of sterols, might be expected to have a much lower permeability to water and other ions than general body membranes. We believe this is related to the very large surface area of the cilia. The primary cilia function appears related to propulsion and water flow across the gills. However, their mere presence will increase substantially an organism's surface area. Afzelius (19) examined another ctenophore species similar in size to those studied here and, based on his values for length and number of cilia, we estimate that the surface area of the cilia may be over twice the body area. This clearly would pose considerable problems in controlling water and ionic fluxes across the surface of the organism. Our data on the lipid composition of cilia suggest that the solution has been achieved by constructing the ciliary membranes so that they are extremely impermeable and hence take little part in normal exchange processes.

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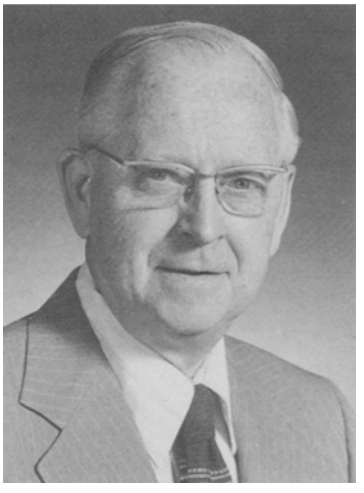
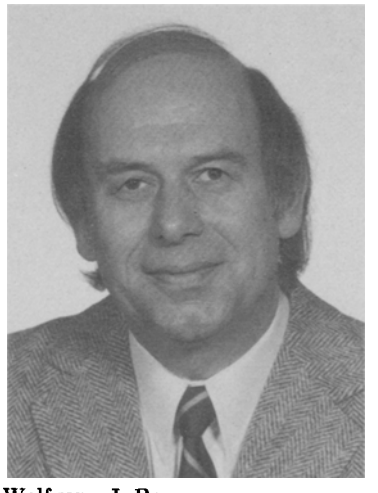
ERRATUM

The symposium "Dietary Factors Affecting Lipid Metabolism," published in the November issue of *Lipids,* was presented at the 74th AOCS annual meeting in Chicago, Illinois. The meeting site was incorrectly listed in the November issue.



Ralph T. Holman



Wolfgang J. Baumann

Editorial

Beginning with the first issue of Volume 21, Dr. Wolfgang J. Baumann will assume the editorship of *Lipids*. He was selected by the Governing Board of the American Oil Chemists' Society to serve as Editor of *Lipids* starting January 1. After that time, the present Editor will serve as Co-Editor.

When *Lipids* was inaugurated as a new journal, separate from the *Journal of the American Oil Chemists' Society*, A. R. Baldwin was Editor of both journals. Beginning with Volume 2, Walter O. Lundberg served as Editor. The present Editor began his service with the July issue of Volume 9. In its first five years, *Lipids* grew rapidly from a bimonthly journal with less than 500 pages to a monthly journal publishing more than a thousand pages.

The subject matter has changed somewhat over the 20 volumes. Analytical methods have changed from macro to micro. Biological studies have gone from whole animals to subcellular particles. Emphasis has changed from fats to biological membranes.

Beginning with Volume 21, the page size of *Lipids* will be enlarged to 8.25 × 11 inches with a two-column format. The *Journal of the American Oil Chemists' Society* and *Lipids* will share new computer-operated typesetting equipment in our production office, and the two journals will have a similar format and type. This will facilitate and economize the production of both journals.

It has been a pleasure to serve as Editor these past eleven and a half years, and I wish to thank the members of the Editorial Advisory Board and the Board of Associate Editors for good advice and for the time and effort spent to produce a peer-reviewed journal. Many thanks are also due to Mrs. Donna Patten, our Editorial Assistant, who will continue in that capacity and who has given years of dedicated service to *Lipids*. The managing editors, James Lyon and George Willhite, and their editorial assistants have been extremely helpful and most cooperative in the production end of the enterprise. I wish to thank also the Hormel Institute and the University of Minnesota for making a home for *Lipids* these past 20 years.

I am confident that under the experienced and enthusiastic leadership of our new Editor, *Lipids* will continue to grow and flourish in the years to come.

Ralph T. Holman
Editor